UNIVERSITY OF ALBERTA

INVASION OF EUCARYOTIC CELLS BY *BORDETELLA* SPECIES

BY

CAROL A. EWANOWICH

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1. Ewanowich, C.A., R.K. Sherburne, S.F.P.Man and M.S. Peppler. 1989. *Bordetella parapertussis* invasion of HeLa 229 cells and human respiratory epithelial cells in primary culture. Infect.Immun. **57**:1240-1247.

2. Woods, D.E., R. Franklin, S.J.Cryz Jr., M. Ganss, M. Peppler and C. Ewanowich. 1989. Development of a rat model for respiratory infection with *Bordetella pertussis*. Infect. Immun. **57**:1018-1024.

3. Ewanowich, C.A., A.R. Melton, A.A. Weiss, R.K. Sherburne, and M.S. Peppler. 1989. Invasion of HeLa 229 cells by virulent *Bordetella pertussis*. Infect. Immun. **57**:2690-2704.

4. Ewanowich, C.A. and M.S. Peppler. 1990. Phorbol acetate inhibits HeLa 229 invasion by *Bordetella pertussis* and other invasive bacterial pathogens. Infect. Immun. **58**:3187-3193.

We would be grateful for your approval to have data from the above papers archived as part of Carol's thesis.

Thank you for your assistance in this matter.

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Reseach, for acceptance, a thesis entitled Invasion of Eucaryotic Cells by *Bordetella* species submitted by Carol A. Ewanowich in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Date: 18 Dec 90

This thesis is dedicated to my husband Daryl,

my parents Walter and Marlene;

to the memory of my mother, Gwendolyn

and my friend Jan

Nothing is rich but the inexhaustible wealth of nature.

She shows us only surfaces,

but she is a million fathoms deep.

-Ralph Waldo Emerson

ABSTRACT

Bordetella pertussis and *Bordetella parapertussis* are causative agents of the human syndrome known as whooping cough. Contrary to the belief that *Bordetella* are noninvasive pathogens, we demonstrate that both of these species can invade eucaryotic cells. Transmission electron microscopic examination of infected HeLa 229 cells revealed evidence of uptake of *B. pertussis* and *B. parapertussis* into tight-fitting phagosomes within hours of inoculation. Evidence of lysosomal fusion was not observed. Comparable numbers of *Bordetella sp.* and invasive isolates of *Salmonella* and *Shigella* were recovered from HeLa monolayers seven hours post-infection.

A comparison of the recovery of several mutants of *B. pertussis* indicated that expression of filamentous hemagglutinin, pertussis toxin and two unidentified proteins whose expression is virulence-regulated were required for maximum invasion. In contrast, expression of adenylate cyclase toxin was correlated with a reduction in invasion and/or survival within HeLa cells.

Peptides containing Arg-Gly-Asp sequences corresponding to two Arg-Gly-Asp sequences found within a newly-recognized 69 kDa outer membrane protein of *B. pertussis* termed pertactin were synthesized. Of these, a 14-mer peptide corresponding to the first Arg-Gly-Asp sequence in the open reading frame of pertactin designated P1 caused a significant reduction in HeLa invasion by *B.*

pertussis. Similarly, preincubation of *B. pertussis* with a monoclonal antibody which recognized P1 also caused a significant reduction in invasion. Peptides containing Arg-Gly-Asp sequences corresponding to tripeptide sequences within fibronectin, filamentous hemagglutinin, and the second tripeptide sequence in pertactin had no effect on invasion.

Evidence for invasion and intracellular survival of *B. pertussis* and *B. parapertussis* was also gleaned from a rat model of respiratory infection in which adult rats were inoculated intratracheally with bacteria encased within agar beads. Viable extra- and intracellular bacteria of both species were recovered from infected rat lungs at 31 days post-infection. Transmission electron microscopic examination of infected lung tissue indicated that viable *Bordetella* were harbored within a phagocytic cell type.

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TABLE OF CONTENTS

Chapter I. Introduction.....1

A. Introduction and rationale for experimentation..1

B. Virulence factors of *Bordetella*.....3

1. Filamentous hemagglutinin.....4

2. Pertussis toxin.....5

3. Adenylate cyclase toxin/hemolysin.....7

4. Agglutinogens.....9

5. Dermonecrotic toxin.....10

6. Tracheal Cytotoxin.....11

7. 69-kilodalton outer membrane protein.....12

C. Control of expression of virulence determinants.....13

1. Antigenic modulation.....14

2. Phase variation.....17

D. Conclusions.....18

E. Bibliography.....19

Chapter II. Characterization of invasion of HeLa 229 cells and human respiratory epithelial cells in primary culture by *B. parapertussis*.....38

A. Introduction.....38

B. Materials and methods.....39

1. Bacterial strains.....39

2. Human respiratory epithelial tissue.....40

3. HeLa cell culture methods and invasion

assays.....41
4. Alteration of HeLa cells.....42
5. Effect of antibodies on internalization...42
6. SDS-PAGE and western blotting.....44
7. Transmission electron microscopy.....45
8. Indirect immunofluorescence detection of FHA.....46
C. Results.....46
1. Uptake comparisons of *B. parapertussis* vs.invasive and noninvasive control organisms.....46
2. Transmission electron microscopic examination of infected monolayers.....48
3. Alteration of HeLa cells with microfilament and RME inhibitors.....49
4. Effect of antisera to Bordetellae surface structures on invasion.....51
5. Demonstration of *B. parapertussis* invasion of human respiratory epithelial tissue...52
D. Discussion.....53
E. Bibliography.....72

Chapter III. Invasion of HeLa 229 cells by virulent *B. pertussis*.....76
A. Introduction.....76
B. Materials and methods.....77
1. Bacterial strains.....77

2. Invasion assays.....77
3. Alteration of HeLa cells.....79
4. Pretreatment of *B. pertussis* strains with FHA.....79
5. Effect of antibodies on internalization..80
6. Quantitation of intracellular cAMP in HeLa cells treated with cholera toxin or forskolin.....80
7. Transmission electron microscopy of HeLa cells infected with *B. pertussis*.....81
C. Results.....82
1. Virulence-dependent uptake of *B. pertussis* by HeLa cells.....82
2. Effect of cyclic AMP on invasion.....84
3. Effect of exogenous FHA on invasion of *B. pertussis* FHA$^-$ mutants.....86
4. Transmission electron microscopic examination of HeLa monolayers infected with *B. pertussis*.....86
5. Entry of *B. pertussis* via a microfilament-dependent process.....87
6. Effect of monoclonal and polyclonal antisera on invasion by *B. pertussis*.....88
D. Discussion.....89
E. Bibliography.....104

Chapter IV. PMA-mediated inhibition of HeLa 229 invasion by B. pertussis and other invasive pathogens.....109

A. Introduction.....109

B. Materials and methods.....112

1. Bacterial strains.....112

2. Fluorescence labelling of F-actin and vinculin.....113

3. Treatment of HeLa cells and invasion assay.....113

4. Adherence assay.....114

C. Results.....114

1. Effects of PMA and cytochalasins B and D on actin distribution.....114

2. Effect of PMA on bacterial invasion of HeLa cells.....115

3. Efficacy comparison of PMA and cytochalasins B and D on inhibition of bacterial invasion.....116

4. Effect of PMA and cytochalasins B and D on adherence of BP338 to HeLa monolayers.....117

D. Discussion.....119

E. Bibliography.....134

Chapter V. Role of a 69-kDa outer membrane protein in the invasion of HeLa cells by

Bordetella pertussis.....140

A. Introduction.....140

B. Materials and methods.....142

1. Bacteria and growth conditions.....142

2. Invasion assays.....143

3. Inhibition of invasion by synthetic peptides.....144

4. Effect of monoclonal antibody F6E5 on invasion.....144

C. Results.....145

1. Effect of synthetic peptides on *B. pertussis* invasion of HeLa cells.....145

2. Effect of a monoclonal antibody to the P1 sequence in pertactin on the invasion of *B. pertussis*.....147

3. Comparison of the abilities of mutants lacking pertactin and/or FHA to invade HeLa monolayers.....147

D. Discussion.....149

E. Bibliography.....165

Chapter VI. Development of a rat model for respiratory infection with *Bordetella pertussis* and *Bordetella parapertussis*.....172

A. Introduction.....172

B. Materials and methods.....174

1. Bacterial strains and growth conditions.174

2. Preparation of inocula.....175
3. Infection procedure.....176
4. Quantitation of viable total and intracellular bacteria.....176
5. Fluorocarbon labelling of phagocytic cells.....178
6. Transmission electron microscopy.....179
7. Histological procedures.....179
C. Results.....180
1. Quantitative bacteriological findings...180
2. Histopathological findings.....181
3. TEM examination of lung tissue infected with BP338.....182
D. Discussion.....183
E. Bibliography.....200

Chapter VII. Discussion.....203
A. Adherence and entry.....204
1. Adherence.....204
2. Role of the host cell in bacterial invasion.....205
3. Bacterial determinants of host cell invasion.....206
B. Survival within the host cell.....210
C. Animal models for *Bordetella* invasion.....215
D. Conclusions.....217
E. Bibliography.....220

LIST OF TABLES

Table

Page

II.1 Comparison of uptake of invasive and non-invasive bacterial strains by HeLa 229 monolayers.....61

II.2 Effect of cytochalasins B and D on internalization of *B. parapertussis* 17903 by HeLa 229 monolayers.....62

II.3 Effect of antisera on internalization of *B. parapertussis* by HeLa 229 cells.....63

III.1 List of strains.....94

III.2 Stimulation of cyclic AMP levels in IBMX-treated HeLa 229 monolayers by cholera toxin and forskolin.....95

III.3 Effect of cholera toxin and forskolin on invasion of HeLa 229 cells by *B. pertussis* adenylate cyclase toxin mutants.....96

III.4 Effect of monoclonal and polyclonal antisera on internalization of *B. pertussis* 338 by HeLa 229 cells.....97

IV.1 Comparison of efficacy of agents used to inhibit bacterial invasion of HeLa 229 monolayers.....124

V.1 The integrin family of adhesion molecules....156

V.2 List of strains.....157

V.3 Synthetic peptides used for inhibition of HeLa cell invasion by *Bordetella pertussis*...158

LIST OF FIGURES

Figure
Page

II.1 Transmission electron micrographs of intracellular *B. parapertussis* 17903 within HeLa 229 monolayers.....64

II.2 Transmission electron micrographs of HeLa 229 monolayers infected with *B. parapertussis*- process of entry.....66

II.3 Recognition of *B. parapertussis* 17903 FHA by purified IgG antibodies raised against anti-*B.pertussis* FHA demonstrated by immunoblotting.....68

II.4 Transmission electron micrographs of human respiratory epithelial cells in primary culture infected with *B. parapertussis*.....70

III.1 Invasion of HeLa 229 monolayers by *B. pertussis* mutants.....98

III.2 Effect of exogenous FHA on invasion of strains BP338, BP353 and BP347.....100

III.3 Transmission electron micrographs of HeLa 229 cells infected with *B. pertussis*.....102

IV.1 Rhodamine-phallloidin fluorescence labelling of F-actin in HeLa 229 monolayers.....125

IV.2 Dose-dependent inhibition of invasion of HeLa 229 monolayers by various invasive bacteria mediated by PMA.....127

IV.3 Effect of mezerein and α4-phorbol-12,13-didecanoate on uptake of BP338 by HeLa cells.129

IV.4 Effect of PMA, mezerein, cytochalasins B and D, and α4-phorbol-12,13-didecanoate on adherence of BP338.....131

IV.5 Schematic representation of physical linkages between integrins, talin, vinculin, α-actinin and F-actin.....133

V.1 Effect of synthetic peptides containing RGD sequences of pertactin, FHA and fibronectin

on the ability of BP338 to invade HeLa cells.159

V.2 Ability of P1-pertactin-specific monoclonal antibody F6E5 to inhibit invasion of HeLa cells by BP338.....161

V.3 Invasion of HeLa 229 cells by *B. pertussis* 18323 strains 868 (parent), 872 (FHA$^-$), 870 (Pn$^-$) and 875 (FHA$^-$, Pn$^-$).....163

VI.1 Viable counts of BP338 (virulent parent) in rat lung.....188

VI.2 Viable counts of BP347 (vir$^-$) in rat lung....190

VI.3 Viable counts of BP353 (FHA$^-$) in rat lung....192

VI.4 Viable counts of BP357 (PT$^-$) in rat lung.....194

VI.5 Viable counts of *B. parapertussis* in rat lung.....196

VI.6 Transmission electron microscopic view of alveolar lung tissue recovered 3 days after intratracheal inoculation with BP338.....198

VII.1 Interaction between the amiphipathic polymyxin B molecule and a biological membrane.....219

Chapter I

INTRODUCTION

A. INTRODUCTION AND RATIONALE FOR EXPERIMENTATION

Members of the genus *Bordetella* are small (approximately 1 x 0.3 μm), fastidious, gram-negative coccobacilli which are strictly aerobic in their oxygen requirements. They were initially designated members of the genus *Hemophilus* by Bordet and Gengou in 1906 (6); however, their genus name was later changed to *Bordetella* when it was discovered that none of its members required either X or V factors for growth (35).

Of the four species comprising the genus, only *B. pertussis* and *B. parapertussis* are considered human pathogens, responsible for the syndrome known as whooping cough. Clinically, the course of classical whooping cough is described in three stages. The initial prodromal period, known as the catarrhal stage, lasts 7-10 days and is characterized by non-specific respiratory symptoms and general malaise. This is followed by the paroxysmal stage which is marked by frequent vomiting and coughing spells. During this stage, forced inspirations over a partially closed glottis following such coughing spells are manifested as the characteristic whoop. Such symptoms gradually diminish over several weeks as the patient enters the convalescent stage. Classical pertussis is the form

most commonly seen in infants and nonimmunized young children, whereas atypical or mild pertussis is more common in immunized children and adults. The incidence of asymptomatic carriage is low and the existence of a true carrier state has not been documented.

The apparent lack of a true carrier state is difficult to reconcile with the fact that humans are the only reservoir for both *B. pertussis* and *B. parapertussis*, especially in cases where infectious contacts are not apparent. One possible explanation for this descrepancy is the existence of *Bordetella* in an intracellular location. Such intracellular organisms would likely not be detected in bacteriologic surveys due to the relatively noninvasive nature of such sampling. This possiblity is also supported by the results of several previous studies which suggest the existence and/or survival of *B. pertussis* within both professional and nonprofessional phagocytes (13, 19)

The first data indicating a possible intracellular location for *Bordetella* was reported by Crawford and Fishel in 1959. (19). These authors described the outgrowth of *B. pertussis* from infected HeLa, KB, DMB (human nasal epithelium), and primary lines of monkey kidney tissue following antibiotic treatment. This was followed in 1969 by a report from Cheers and Gray (13) describing the survival of *B. pertussis* within murine macrophages during the "complaisant" stage of pertussis in which numbers of viable bacteria were kept relatively constant by

readjustments of host immune mechanisms. Finally, Hopewell *et al.* in 1971 observed intracellular *B. pertussis* within the cytoplasm of ependymal cells at the onset of intracerebral infection in a murine model (34). Although the authors indicated that ependymal cells are not known to be actively phagocytic, their discussion did not emphasize the intracellular location of the bacteria because evidence of subsequent intracellar destruction of the bacteria was noted six days post-infection.

Taken together, these reports indicated that intracellular localization and short-term persistence as a means of carriage of *Bordetella* was a viable possiblity worthy of further study. Such was the impetus for the experimentation presented in this thesis.

B. VIRULENCE FACTORS OF BORDETELLAE

Bordetella pertussis and *Bordetella parapertussis* produce an impressive array of virulence determinants whose synergistic interaction mediates host colonization and pathology. The expression of most of the virulence factors produced by *Bordetella* is positively regulated in *trans* by a gene product known as Vir (85), or more recently, Bvg (72).

1. Filamentous Hemagglutinin

Filamentous hemagglutinin (FHA) is a nonfimbrial protein expressed by virulent *Bordetella* that agglutinates a variety of erythrocytes (1, 74, 89) Physically, FHA is an aggregate of hydrophobic filamentous structures measuring 2 nm in diameter and 40-100 nm long (74). The major bands seen on sodium dodecyl-sulfate-polyacrylamide gel electrophoresis are 100,000, 130,000 and 200,000 molecular weights (17) which probably represent polymers or degradation products of the native protein. The hemagglutinating activity of FHA is sensitive to inhibition by cholesterol (74).

Biologically, FHA is believed to act as an attachment factor, mediating adhesion of *Bordetella* to mucosal surfaces in the respiratory tract (80, 82). In addition, FHA constitutes at least one component of *B. pertussis* which promotes adherence of these bacteria to several mammalian cell lines, including HeLa (68, 73) Vero (30) and WiDr cells (83). More recently, FHA has been reported to bind to human macrophages using two distinct receptors. It appears to interact with both the β2 integrin CR3 receptor via an Arg-Gly-Asp sequence at positions 1097-1099, as well as a class of galactose-containing glycocongugates found on human macrophages (69). Its importance as a protective antigen has been demonstrated by studies in which systemic immunization with FHA provides significant protection against challenge with *B. pertussis* in mouse respiratory

infection models (39, 60). Consequently, FHA is a major candidate for inclusion in component acellular vaccines currently under study.

2. Pertussis Toxin

Pertussis toxin is a hexameric molecule consisting of five dissimilar subunits designated S1-5, with a total molecular weight of approximately 105 000 (47, 57). In the native toxin, the S4 subunit is represented twice, forming two different dimers with S2 and S3 (78). The classical A-B structure of PT was assigned by Tamura *et al.*, with the enzymatically active S1 subunit designated the A (Active) protomer, and the remaining pentamer the B (Binding) oligomer (78).

While the B subunit of PT targets the toxin to receptors on susceptible host cells, the A-protomer acts as an ADP-ribosyltransferase which catalyzes the transfer of ADP-ribose from NAD to the α subunit of certain membrane-embedded guanine nucleotide-binding proteins (G proteins) which act as a transmembrane linkage coupling extracellular receptors to the intracellular adenylate cyclase enzyme (38, 62). As a result of this covalent modification, the inhibitory G-protein (G_i) can no longer hydrolyze bound GTP, rendering the cell unresponsive to inhibitory hormonal signals. This does not directly result in an increase in cellular cyclic AMP. However, once the cyclase is activated by an extracellular regulatory molecule, the inhibitory

activities of the toxin-modified G-proteins can do little to check the activity of the activated cyclase enzyme. (38, 42).

It should be mentioned that not all toxin activities are due to modified adenylate cyclase regulation, and other pathways may be affected. For example, pertussis toxin has been reported to inhibit receptor-mediated release of arachidonic acid (5), inhbition of hydrolysis of phosphatidyl inositol (8) and calcium mobilization (48, 55). The wide variety of biological effects produced by pertussis toxin has generated an array of synonyms by which the toxin is also known, including pertussigen, islet-activating protein, histamine-sensitizing factor, and lymphocytosis-promoting factor.

Of the four members of the genus, expression of pertussis toxin is limited to *B. pertussis* only. Although *B. parapertussis* and *B. bronchiseptica* contain the genes encoding pertussis toxin, the genes contain clusters of base pair substitutions within the promoter of S1 which likely severely limit its efficiency and impair its recognition by the *trans*-acting regulatory element encoded by the *vir* locus (2, 49).

In experimentally infected mice, PT causes multiple metabolic disturbances including hyperinsulinemia (31) and hypoglycemia (77), histamine sensitization (54), lymphocytosis (53), and inhibition of macrophage and neutrophil migration (50). In humans, PT is responsible for

lymphocytosis, hypoglycemia, and occasionally neurotoxicity (64). PT is also thought to act as an adhesin of *B. pertussis*, mediating adherence of the organisms to ciliated respiratory cells, as demonstrated both *in vitro* with ciliated respiratory epithelial cells isolated from humans (82), and *in vivo* in a rabbit model of pertussis (81).

3. Adenylate Cyclase Toxin/Hemolysin

Bordetella species secrete a bifunctional protein which encodes both adenylate cyclase toxin (ACT) and hemolysin activities (26). Whereas the physical and biological properties of the hemolysin are not well characterized as yet, properties of the adenylate cyclase toxin have been intensely studied since its discovery in 1973 (87).

ACT has been recently cloned and sequenced (26). The calculated molecular weight of the complete unmodified transcript is 177 kDa (26), which is in agreement with the 200 kDa form of the enzyme reported to be capable of penetrating target cells and generating large increases in intracellular cyclic AMP from endogenous ATP (33, 71). ACT is activated by calmodulin, a eucaryotic regulatory calcium-binding protein (88). This is unusual in light of the fact that procaryotes generally do not possess calmodulin, and, coupled with the observation that the enzyme is actively secreted by the bacteria during

exponential growth (32) further points to its role as a bacterial toxin (86)

The net effect of an uncontrolled increase in intracellular cyclic AMP in target cells intoxicated by ACT appears to be immune impairment. Confer and Eaton first reported that urea extracts of *B. pertussis*, containing active ACT, elicited large increases in cyclic AMP in human neutrophils and alveolar macrophages which were associated with reduced superoxide-mediated killing of *Staphylococcus aureus* (15). Increases in cyclic AMP generated by ACT activity have since been reported to impair other effector mechanisms of phagocytic cells including chemiluminescence, bacterial killing, phagocytosis, chemotaxis, and phagolysosome fusion (A. M. Friedlander *et al.*, Fed. Proc. 42:3360, 1983, 24, 25, 63). Functional interference of non-phagocytic cell types by ACT has also been reported, including the inhibition of cytolytic activity of natural killer lymphocytes (P. Symes, E. L. Hewlett, D. Roberts, A. Q. de Sousa and R. D. Pearson, Clin. Res. 1983, p. 377A)

Immune interference by ACT is thought to contribute to the pathogenesis of *Bordetella* by paralysis of host clearance mechanisms. This would facilitate both establishment of *Bordetella sp.* and superinfection by any other opportunistic pathogens which would otherwise be cleared in the absence of ACT-induced immunological impairment. The importance of ACT as a virulence factor *in*

vivo was demonstrated in a report by Weiss *et al.* in 1983 in which a strain deficient in ACT was avirulent in a murine model (84). A later study by Goodwin and Weiss (29) indicated that adenylate cyclase was required for establishment of virulent *B. pertussis* in infant mice. Such data generated from *in vivo* experimentation further underlines the importance of ACT in *Bordetella* pathogenesis.

4. Agglutinogens

Agglutinogens, or surface determinants that elicit antibodies that can agglutinate whole bacteria, are used as the basis for serological classification of *Bordetella* (21). Of the 14 possible agglutinogens, *B. pertussis* contains 8, of which 1-6 are unique to the species. Agglutinogen 1 represents, at least in part, specific determinants on the lipooligosaccharide of the the bacteria and are common to all *B. pertussis* (45). Agglutinogens 2 and 3 are fimbrial in nature (4, 37, 89) (although there exists some disagreement regarding the fimbrial nature of agglutinogen 3 [11, 18]) and are expressed in various combinations on the surfaces of virulent bacteria. Of the various combinations possible, four serotypes are recognized: 1,2,3; 1,2; 1,3; and 1 (67). Of these four, only serotypes containing agglutinogen 1 in combination with 2 or 3 are known to infect man (65), an observation

which points to a significant role for agglutinogens 2 and 3 in pathogenesis.

Although antisera raised against agglutinogens appear to inhibit adherence of *B. pertussis* to various types of non-ciliated tissue culture cells including Vero (30) and HeLa cells (68), they are not apparently required for adherence to human ciliated respiratory cells (82), their natural target.

Despite the lack of data indicating their importance as adhesins, agglutinogens are still considered potential candidates for components of acellular vaccines based on *in vivo* animal protection studies and epidemiological data. Agglutinogens actively protect mice against intranasal infection in the mouse (70), and human antibodies against agglutinogens afford mice passive protection against aerosol infection (61). In addition, infection by type 1,3 organisms in children vaccinated with type 1,2 strains and *vice versa* have been reported in epidemiological studies (36, 66). Consequently, the World Health Organization currently recommends that strains used for whole-cell vaccines should contain agglutinogens 1, 2 and 3.

5. Dermonecrotic Toxin

Dermonecrotic toxin (DNT), heat-labile or mouse lethal toxin, first described by Bordet and Gengou in 1909, is a simple protein with an apparent molecular weight of 102 kDa (22). The reported biological activity of the toxin

appears to depend chiefly on the purity of the preparation tested, with effects ranging from hemorrhagic necrotic responses in suckling mice injected subcutaneously with limited doses and lethal activity in larger doses (16, 46), to ischemia and edema in some cases (41). Its principal target tissue *in vivo* appears to be vascular smooth muscle, which suffers vasoconstriction within minutes following application of the toxin and, hours later, consequent tissue ischemia and leukocyte diapedesis (23). In contrast with its severe toxic ability *in vivo*, DNT has no apparent effects on cultured cells or tissues studied in isolation from the host.

Due to its vasoconstrictive activity, DNT is thought to exacerbate the symptoms of human whooping cough principally in the catarrhal stage, acting on highly vascularized tissues where it elicits rhinorrhoea, sneezing, swelling of the eyelids, conjunctival congestion, and resultant inflammatory responses (56).

6. Tracheal Cytotoxin

Tracheal cytotoxin (TCT) is a small peptide with structural similarity to peptidoglycan, containing both diaminopimelic acid and muramic acid (27). TCT appears to be enzymatically released from native *B. pertussis* peptidoglycan (28). *B. parapertussis*, *B. bronchseptica* and *B. avium* also release a chemically identical TCT molecule during log-phase growth (14). In hamster tracheal organ

culture, purified TCT causes a reproducible, progressive pattern of cytopathology spanning several days, depending on the concentration of TCT tested. At specific concentrations of TCT, ciliary activity ceases within 72 hours of application. This is followed by death and extrusion of ciliated cells within 96 hours, while non-ciliated cell types remain ultrastructurally normal (27). In cultured hamster tracheal epithelial cells, application of TCT causes inhibition of DNA synthesis in a dose-dependent fashion (Goldman, W. E. and J. B. Baseman, Abst. Ann. Meet. Amer. Soc. Microbiol. 1980, p. 49). The pathological consequences of an inhibition of DNA synthesis seem unclear in light of the fact that mature, surface-exposed ciliated cells do not themselves divide. It has been speculated that such inhibition may inhibit ciliated-cell regeneration due to an underlying toxicity for the epithelial basal cell population (28). Such inhibition may be responsible for continuing respiratory tract pathology following eradication of bacteria, in addition to interference with normal lung defenses which would invite superinfection by other respiratory pathogens.

7. 69-kilodalton Outer Membrane Protein

A newly recognized 69-kilodalton non-fimbrial outer membrane protein of *B. pertussis* was initially described by Brennan *et al.* in 1988 (9). Like other agglutinogens, it elicits the production of agglutinating antibodies in mice

(9). Like other agglutinogens, except agglutinogen 1 (Los A), it is expressed only by virulent phase organisms (12) (see pages 13-17 for an explanation of virulence determinant expression by Bordetellae). Antigentically similar versions of this protein, with slightly different molecular weights, are also found on *B. parapertussis* and *B. bronchiseptica*.

Interest in this protein was sparked following several reports of its efficacy as a protective antigen in various animal models (10, 52, 59, 75), In addition, the 69 kDa antigen can induce B cell-mediated responses in children immunized with either whole-cell (79) or acellular vaccines (75), as well as T-cell mediated immunity in a convalescent adult (20). Due to its potential as a protective antigen, intense effort is currently being directed towards extensive characterization of the protein as both an antigen and virulence determinant of *B. pertussis*.

C. CONTROL OF EXPRESSION OF VIRULENCE DETERMINANTS

Expression of each of the virulence determinants outlined above can be modified by either of two distinct processes, with the single exception of TCT, whose expression is unaffected by either.

1. Antigenic Modulation

Antigenic or phenotypic modulation decribes a reversible shift between alternative virulence phenotypes induced by various environmental stimuli which occurs in all, or nearly all, members of a population exposed to the stimulus. For example, growth at low temperature (<28°C), or in the presence of various salts or fatty acids results in a reversible loss in expression of virulence determinants; a shift from the so-called X-mode (virulent phase) to the C-mode (avirulent phase) (43).

Differential expression of the central regulatory locus designated *bvg* (*B*ordetella *v*irulence *g*ene), originally termed *vir* (84, 85), modifies expression of several unlinked genes and operons encoding virulence factors that comprise the *bvg* regulon. It is this central regulatory locus which coordinately regulates the expression of *bvg*-activated genes in response to various environmental conditions, acting through a signal transduction mechanism similar to that found in various other highly adapted pathogens (2, 51).

The *bvg* locus contains three tandem open reading frames designated *bvgA*, *bvgB* and *bvgC* (3). Based on nucleotide sequence determination, the predicted products of *bvgA* and *bvgC* bear significant sequence homology to members of the two-component sensor/regulator family of bacterial sequence transduction proteins (3). The BvgA protein shares homology with known regulator components due

to a specific N-terminal receiver domain, while BvgC, with a specific C-terminal transmitter domain, appears to function as the sensor component (51, 58), although it contains an additional receiver module at its C-terminus whose function is unclear (3). BvgB contains neither a transmitter nor a receiver domain, yet is still required for regulation (72). A putative model outlining the interactions between *bvg* products has been proposed by Arico *et al.* (3). In this model, BvgB and the N-terminal domain of BvgC are located in the periplasmic space, and BvgA in the cytoplasm. Environmental signals are sensed by the C-terminal transmitter module of BvgC, which in turn transduce signals to the corresponding receiver module in the N-terminus of BvgA, thereby modifying the capacity of BvgA to act as a transcriptional activator (72) of various genes encoding virulence determinants. The transmitter domain of BvgC is proposed to possess potein kinase activity which activates BvgA by phosphorylation, although proof of such protein kinase activity has yet to be documented. In addition to the set of genes whose expression is inhibited in the presence of the modifying signals outlined above (ie. the absence of *bvg* transcription), the so-called *vir*-activated genes, Knapp and Mekalanos (40) have identified an additional set of genes which are expressed in the presence of such modifying signals due to the resultant absence of *bvg* gene products. To date, the functions of vir-repressed genes are unknown.

An alternate model of signal transduction by *vir* gene products was recently proposed by S. Stibitz (S. Stibitz and M. S.-Yang, *Abstr. Sixth Int. Symp. Pertussis*, 1990, p. 57). Cellular localization of *vir*-gene products fused with alkaline phosphatase indicated that BvgA is cytoplasmic (Pho^-) and Bvg B is periplasmic (Pho^+), consistent with the model described above. Unlike the previous model, however, the Pho^- phenotype of BvgC predicts a cytoplasmic location. Stibitz and Yang proposed that BvgB and BvgC instead constitute a single periplasmic protein capable of sensing changes in the external environment of the bacterium. Due to its sensory capacity, they termed this protein BvgS (S, sensory). In addition, prelimary data suggested a role for dimerization of adjacent BvgS proteins in signal transduction to the periplasmic receiver domain of BvgA.

The ability of *Bordetella* to self-regulate expression of virulence determinants depending on changing environmental conditions would lend a definite adaptive advantage to these organisms. For example, down-regulation of expression of virulence determinants (such as attachment factors) in later stages of infection which were required in the initial stages for adequate establishment of the bacteria could facilitate both establishment of a carrier state and transmission to new hosts (51).

2. Phase Variation

Phase variation is the process by which members of the genus *Bordetella* shift between distinct metastable genetic states, or phases. At one end of the spectrum is the virulent phase, in which all virulence-associated determinants are fully expressed, while at the opposite end is the avirulent phase, where the synthesis of virulence factors is fully repressed (44). Populations of *Bordetella pertussis* undergo phase variation from a virulent to an avirulent phase with a frequency ranging between 10^{-3}-10^{-6}, depending on the strain (35, 76). A similar frequency is observed for a shift in the opposite direction (M. S. Peppler, unpublished observations).

Although it was known for some time that a product of a regulatory locus designated *vir* was required for expression of many virulence-associated loci (85), the precise mechanism of phase variation and the role of *vir* was unclear. The answer came in 1989, when Stibitz *et al.* reported that phase variation in one series of strains reflected a simple single base-pair frameshift mutation within an open reading frame which was predicted to code for a *vir* product (76). The mutation was pinpointed by comparison of DNA sequences between corresponding restriction fragments obtained from isogenic virulent and avirulent pairs of *B. pertussis*. A single additional cytosine residue was detected in avirulent strains which was absent from the corresponding virulent pairs at

position 4129 of *bvgC*, (or, more correctly, *bvgS*) one of the three open reading frames within the *bvg* locus. It is not known yet whether frameshift mutations at this specific location are entirely random, or whether they are site-specific events which play a significant role in the adaption of *Bordetella* to changed environments.

D. CONCLUSIONS

Bordetella sp. are highly adapted pathogens which express an impressive array of specialized virulence determinants. These virulence factors interplay, with some degree of redundancy, to produce a highly complex disease in a manner which is still incompletely understood. In addition to our lack of a clear understanding of the complex and multifactorial nature of pathogenesis caused by *Bordetella*, our understanding and appreciation of the mechanisms these organisms have evolved to permit appropriate expression of virulence determinants is still in its infancy. In the not-so-distant past, whooping cough was regarded as little more than a toxicosis. We are only now beginning to appreciate the nuances of pathogenesis of Bordetellae.

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Chapter II

CHARACTERIZATION OF INVASION OF HELA 229 CELLS AND HUMAN RESPIRATORY EPITHELIAL CELLS IN PRIMARY CULTURE BY *BORDETELLA PARAPERTUSSIS*[1]

A. INTRODUCTION

Within the genus *Bordetella*, the species *Bordetella pertussis* is most frequently associated with cases of whooping cough. Despite the fact that *B. parapertussis* does not synthesize pertussis toxin (1, 10), which is likely responsible for numerous systemic manifestations of whooping cough, this species is also capable of causing disease in humans. The symptomatology of disease caused by *B. parapertussis* is similar to that of *B. pertussis*, albeit generally milder in terms of severity. However, cases of severe infections with *B. parapertussis* involving fatal bronchopneumonia have been reported (9, 20).

In our attempt to critically examine *Bordetella* for evidence of invasive ability, we began with *B. parapertussis* because it was significantly less toxic than *B. pertussis* to eucaryotic cells during prolonged coincubation periods. In this chapter we characterize invasion of both HeLa 229 cells and human respiratory

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[1] A version of this chapter has been published. **Ewanowich, C. A., R. K. Sherburne, S. F. P. Man and M. S. Peppler.** 1988. Infect. Immun. 57:1240-1247.

epithelial cells in primary culture by *B. parapertussis*. The number of viable intracellular *B. parapertussis* within HeLa cells after a defined interval was compared with several known invasive and non-invasive bacterial species, in order to determine whether the numbers of viable *Bordetella* were significant, and to provide a basis for further experimentation.

B. MATERIALS AND METHODS

1. Bacterial Strains.

Bordetella parapertussis 17903 was originally obtained from the Michigan Department of Public Health, Grand Rapids. Organisms were routinely passaged on Bordet-Gengou agar (BGA) containing 13% sheep blood. Positive control strains for invasion included fresh clinical isolates of *Shigella flexneri* and *Salmonella hadar* which were obtained from University of Alberta Hospitals Microbiology Laboratory. *Yersinia pseudotuberculosis* Type 1A and *Yersinia enterocolitica* serotype 0:3 strain E2549 were provided by the Enteric Division of the Provincial Laboratory of Public Health, Edmonton, Alberta. Virulence of *Shigella flexneri* mediated by a) chromosomal and b) plasmid-encoded determinants was ascertained by the presence of a) O-side chain on LPS as seen in SDS-PAGE gels visualized by the silver staining technique of Tsai and Frasch (15), and b) by dye binding ability on Congo Red

agar (3), respectively. Frozen stock cultures were passaged once only on Trypticase Soy Agar (TSA) prior to use in invasion assays in order to minimize reduction in virulence due to plasmid loss. Negative invasion controls included *Escherichia coli* strain 10418 (highly piliated), 10407 (non-piliated variant obtained from the laboratory of Dr. Glen Armstrong, University of Alberta) and *Escherichia coli* strain SA 1377 (rough LPS mutant obtained from Dr. K. Sanderson, University of Calgary), passaged routinely on TSA.

2. Human Respiratory Epithelial Tissue

Following surgical removal, nasal turbinates were washed in physiological saline to remove excess blood. Samples were placed in Eagle's medium (MEM) supplemented with penicillin (50µg/mL), streptomycin (50µg/mL) and gentamicin (50µg/mL), and 0.1% protease (type XIV, Sigma). After 20-24 hours of cold protease treatment at 4°C, mechanical agitation was used to free epithelial cells from the specimen. Fetal bovine serum (FBS) was added to a final concentration of 10% to neutralize the protease. The detached cells were filtered through a 60µM Vitex mesh, centrifuged, and washed once with MEM plus 10% FBS and once in MEM plus 5% FBS prior to being plated in plastic culture dishes on glass coverslips that had been coated with a collagen gel. Collagen gels were prepared from rat tail collagen (type VII, Sigma) according to the method of

Yang *et al.* (19). A cell density of 10,000 cells/cm^2 was used. Confluence was achieved in 3-4 days.

3. HeLa Cell Culture Methods and Invasion Assays.

The established HeLa 229 human epithelia-like cell line (ATCC CCL 2.1) was maintained in Eagle's Minimal Essential Medium (MEM, Gibco Laboratories, Grand Island, N.Y.) containing 5% fetal bovine serum (FBS) (Gibco) without antibiotics in an atmosphere of 5% CO_2. Confluent stock monolayers were used to seed TC-25cm^2 flasks (Corning, Corning, N.Y.) at a concentration of 9 x 10^5 cells per flask. Following overnight incubation, the semiconfluent monolayers were infected with organisms which had been resuspended in MEM/FBS to an optical density of 0.15 at 540 nm and diluted to obtain an appropriate multiplicity of infection (MOI). Due to its relatively lengthy lag phase and generation time in MEM, numbers of *B. parapertussis* do not significantly increase during the five hour coincubation period. In contrast, the enteric species used is this study possess a relatively short lag phase and generation time, and consequently increase in numbers by several logs during the coincubation period. In an attempt to compensate for the differences in generation times, 10-fold fewer *Yersinia sp.*, *Shigella*, *Salmonella* and *Escherichia* than *B. parapertussis* were initially added. MOI's were confirmed retrospectively for each experiment by viable counts. Organisms and HeLa cells were coincubated

for 5 hours, washed twice, then reincubated for 2 hours in the presence of 100µg/mL gentamicin (Gibco) to inactivate extracellular organisms. Viable intracellular organisms were recovered from trypsinized monolayers followed by lysis in distilled water and sedimentation at 7,700 x g and enumerated by plating appropriate dilutions in triplicate onto freshly-prepared TSA or BGA.

4. Alteration of HeLa cells.

Effects of cytochalasins on internalization were determined by preincubation of HeLa monolayers with cytochalasins B and D (Sigma) at concentrations of 1.0 and 2.5 µg/mL containing final concentrations of DMSO of 0.2 and 0.5% respectively. Control monolayers were preincubated with 0.5% DMSO. Viability of cytochalasin-treated monolayers compared with controls was determined by trypan blue exclusion following a total incubation time of 8 hours. Monolayers were similarly treated with monodansylcadaverine (MDC, Sigma) solubilized in MEM by addition of 1N HCl and adjusted to concentrations of 100, 200 and 300 µM in MEM/FBS prior to infection. Invasion assays in the presence of inhibitors were performed as described above.

5. Effect of Antibody on Internalization.

Anti-filamentous hemagglutinin - The purified IgG fraction of goat anti-FHA was kindly provided by Drs. Jim

Cowell and Michael Brennan, Division of Bacterial Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland. These antibodies have been previously shown to inhibit adherence of *Bordetella pertussis* Tohama phase 1 to WiDr cells, an epithelium-like cell line derived from a human intestinal carcinoma (17). The IgG fraction was diluted with Hanks balanced salt solution containing 25mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2) (Hanks-HEPES) and added to suspensions of *Bordetella parapertussis* 17903 (approximately 1 x 10^9 bacteria per mL) to obtain final protein concentrations of 0.25 and 0.50 mg/mL. Due to a lack of the most appropriate control antiserum (purified pre-immune goat IgG antibodies), control organisms were instead incubated in Hanks-HEPES with normal rabbit serum added to a final serum protein concentration of 0.50 mg/mL. After incubation at 37°C for 60 min, bacteria were diluted in MEM/FCS and used in invasion assays as described above.

Anti-*B. parapertussis* and *anti-B. pertussis* - Rabbit antisera raised against *B. parapertussis* 17903 and *B. pertussis* 2231 were provided by Dr. Jack Munoz. Monovalent Fab fragments were isolated from purified IgG fractions following overnight incubation with papain (Boehringer Manneheim, Dorval, Quebec) by passage through a Protein A-Sepharose column (Sigma) to remove Fc fragments. Completion of cleavage by papain was comfirmed by SDS-PAGE. Fab fragments were added to suspensions of *B. parapertussis*

17903 diluted in Hanks-HEPES to obtain final protein concentrations of 0.25mg/mL and used as outlined above.

6. SDS-PAGE and Western Blotting.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% acrylamide slab gels was performed according to the method of Laemmli (8). Bacteria from 3-day-old BGA cultures were suspended in 50mM Tris-63mM glutamate-43mM saline buffer (pH 7.4) to an absorbance of 0.12 optical density units at 540 nm. 1.5 mL was centrifuged at 8,000 x g and the cell pellet was resuspended in Laemmli digestion buffer with dithiothreitol to a final volume of 50µL. Samples were boiled and 10µl aliquots loaded per lane. Proteins were electrophoretically transferred to nitrocellulose (2) overnight at 27V in a 25 mM sodium phosphate buffer, pH 7.4. Membranes were blocked with phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20 (PBST) for 2 hours at 37°C, incubated with goat anti-FHA diluted in PBST for 2 hours at 37°C, then washed extensively with PBS. Alkaline phosphatase-conjugated rabbit anti-goat antisera (Sigma) was diluted in PBST and incubated with blots at 37°C for 1 hour. After extensive washing with PBS, bands were visualized with color development reagents 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP, Bio-Rad Laboratories, Mississauga, Ont.), 15 mg/ml in *N',N'*-

dimethylformamide (DMF), and p-nitro blue tetrazolium chloride (NBT, Bio-Rad), 30 mg/mL in 70% DMF.

7. Transmission Electron Microscopy.

Transmission electron microscopic examination of infected tissues was performed by Richard K. Sherburne, Department of Medical Microbiology and Infectious Diseases, Edmonton, Alberta, Canada.

HeLa monolayers - sparsely confluent HeLa 229 monolayers were infected with *B. parapertussis* 17903 at an MOI of approximately 1, confirmed retrospectively by viable counts. At intervals of 24, 48, and 72 hours, infected and control uninfected monolayers were washed twice with a buffered EDTA solution, fixed *in situ* with 3.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2, at 4°C for 1 hour. Monolayers were washed in cold cacodylate buffer 2 x 15 minutes, then fixed with 1%OsO_4 in cacodylate buffer for 1 hour and recovered by mechanical disruption. The cells were washed 2 x 15 minutes with buffer, dehydrated with a graded series of ethanol (25%, 50%, 75% and 90%), transferred to propylene oxide for 30 minutes. and finally to a 1:1 mixture of propylene oxide and LX112 resin for 24 hours (unstoppered). This mixture was then replaced with LX112 and cured at 60°C for 24 hours. Sections of embedded cells were examined using a Philips transmission electron microscope model 300 or a model 410 equipped with a goniometer stage.

Human respiratory epithelium - confluent monolayers cultured on collagen gel-coated glass coverslips were initially infected with *B. parapertussis* 17903 at MOI's of 1 and 10. Following an 18 h coincubation period, monolayers were washed twice and fixed *in situ* with 3.5% glutaraldehyde in PBS pH 7.3 overnight at 4°C, washed twice in PBS and fixed with 1% OsO_4 in PBS for 1 hour. Intact monolayers were washed twice in PBS, dehydrated, embedded, sectioned and examined as described above.

8. Indirect Immunofluoresence Detection of FHA.

Surface-exposed FHA on methanol-fixed organisms was labelled for fluoresence visualization by sequential incubation with goat anti-FHA, rabbit anti-goat IgG (Sigma) and goat anti-rabbit IgG FITC conjugate (Sigma).

C. RESULTS

1. Uptake Comparisons.

The relative numbers of *B. parapertussis* organisms internalized by HeLa 229 monolayers were compared with control invasive and noninvasive genera (Table II.1). Significantly fewer *B. parapertussis* invaded monolayers compared with *Yersinia enterocolitica* serotype 0:3 and *Yersinia pseudotuberculosis* type 1A. However, values for *B. parapertussis* rival those obtained for the other invasive

control organisms, *Salmonella hadar* and *Shigella flexneri*. No significant difference exists between values for *B. parapertussis* and *Salmonella hadar*, although *B. parapertussis* invaded monolayers with a significantly greater efficiency than the control strain of *Shigella flexneri* ($P < 0.0005$, student's *t*-test). No obvious effects on monolayer integrity caused by invasive control strains were apparent following the 5 h coincubation period as determined by trypan blue staining. Despite efficient binding of the highly piliated *Escherichia coli* negative control strains 10418 and SA 1377 to HeLa monolayers based on light microscopy, recoveries of viable organisms were significantly lower than recovery of *B. parapertussis* ($P < 0.0005$) Lower still was recovery of nonpiliated negative control *E. coli* strain 10407P.

In separate experiments, exposure to gentamicin at a concentration of 100µg/mL for 2 hours was found to inactivate 99.99% of an initial concentration of *B. parapertussis* of 1×10^{10} bacteria per mL. Furthermore, minimum inhibitory concentration determinations on inoculum *B. parapertussis* and organisms recovered from gentamicin-treated monolayers demonstrated identical sensitivities, indicating that viable organisms recovered after antibiotic treatment did not represent a resistant population selected by gentamicin. Viable intracellular organisms could be recovered from monolayers incubated in the continued

presence of gentamicin at least 10 days following initial infection.

2. TEM examination of infected HeLa monolayers.

The data presented above indicating the intracellular presence of viable *B. parapertussis* were confirmed by transmission electron microscopic (TEM) examination of infected HeLa monolayers. Sparsely seeded HeLa monolayers were initially infected with *B. parapertussis* at an MOI of 0.1 and coincubated for intervals of 24, 48 and 72 hours post infection (PI). At each interval, duplicate monolayers were either fixed and processed for microscopic examination or treated with gentamicin for quantitation of viable intracelluar bacteria. At 24 hours PI, random examination of ultrathin sections prepared from these monolayers demonstrated very few intracellular or extracellular adherent organisms. By 48 hours PI, numerous adherent extracellular organisms were present, although few were seen free within the surrounding media. Electron microscopic examination revealed a moderate number of intracellular organisms bound by tight endocytic vacuoles (Fig II.1 A-B). In some instances, organisms appeared free within the cytoplasm; however, when later examined using a tilting goniometer stage apparatus, endocytic vacuoles in contact with the outer membranes of most of these organisms were observed. Numbers of viable intracellular bacteria per

cell remained relatively constant. Heavy infection of monolayers occurred by 72 hours PI. TEM examination demonstrated the presence of large numbers of adherent bacteria per cell, with a similar increase in numbers of intracellular organisms (Fig. II.1 C). Most bacteria occurred singly within tight endocytic vacuoles; vacuoles containing two or more bacteria (some undergoing fission) were occasionally seen.

In order to facilitate visualization of the entry process, we coincubated HeLa monolayers and *B. parapertussis* with a higher initial MOI (10) for a 24 hour period. TEM examination demonstrated organisms in various stages of internalization (Fig. II.2). Here organisms were seen entering HeLa cells through an endocytic process, embraced by outstretched microvilli of the HeLa cell which ultimately met and fused, reforming the continuous plasma membrane of the cell while directing the bacterium into the resultant phagosome. Although numerous clathrin-coated pits were observed associated with the plasma membrane, none were seen in association with endocytic vacuoles surrounding ingested bacteria.

3. Alteration of HeLa cells.

The TEM results suggested that receptor-mediated endocytosis did not contribute to entry of *B. parapertussis*. This interpretation was supported by the use

of monodansylcadaverine (MDC), a known inhibitor of this process (14). Monolayers of HeLa cells were preincubated for 1 hour with MDC at concentrations of 100-400μM. The monolayers were subsequently challenged with *B. parapertussis* in the continued presence of MDC using the standard invasion assay. No significant reduction in numbers of intracellular viable organisms was observed, further suggesting that receptor-mediated endocytosis is probably not involved in uptake of *B. parapertussis* into HeLa cells (data not shown).

The role of the host cytoskeleton in internalization of *B. parapertussis* was evaluated using cytochalasins B and D, inhibitors of particle phagocytosis that disrupt microfilament function. The protocol used in the standard invasion assay was followed after monolayers were preincubated for 1 hour in the presence of cytochalasin B or D at concentrations of 2.5 or 1.0 μg/mL. Due to the reversibility of their actions, concentrations of cytochalasins were maintained throughout all assays. HeLa cells treated for 8 hours with cytochalasins showed profound morphological changes. As shown in Table II.2, exposure of monolayers to cytochalasin D resulted in a dramatic reduction in the numbers of organisms internalized. A similar reduction, although less pronounced, occurs following treatment of monolayers with cytochalasin B. In separate experiments, exposure to either

cytochalasin (2.5µg/mL) for a 5 hour period was shown to have no effect on viability of *B. parapertussis* based on recovery comparisons of viable bacteria. In one representative experiment, untreated control bacteria (0.5% DMSO) numbered 2.1 x 10^8 CFU/mL after the 5 hour coincubation period, while 2.3 x 10^8 CFU/mL and 2.2 x 10^8 CFU/mL were recovered from the bacterial suspensions containing 2.5 µg/mL cytochalasins B or D, respectively.

4. Effect of antisera to surface structures of Bordetellae on invasion.

We used hyperimmune serum raised against filamentous hemagglutinin (FHA), a putative ligand of *B. pertussis* which mediates adhesion of these bacteria to a variety of cell types (including HeLa), to determine whether it could inhibit or reduce invasion of *B. parapertussis* by preventing initial attachment. Goat anti-FHA diluted to 1/1000 (highest dilution tested) was first shown to recognize FHA in both phase 1 *B. pertussis* 3779$^+$ and *B. parapertussis* 17903 in western blots probed with alkaline-phosphatase-conjugated rabbit anti-goat immunoglobulins (Figure II.3). It was interesting to note that initial attempts to demonstrate antibody specificity using ^{125}I-protein A as a probe failed due to the relatively inefficient binding of protein A to caprine IgG (9). Accessibility of anti-FHA to native FHA exposed on the

surface of *B. parapertussis* was also confirmed by indirect immunofluoresence. Having confirmed the ability of this antisera to bind FHA in a species of *Bordetella* other than that against which it was raised, we sought to determine whether its presence could protect HeLa cells from invasion by *B. parapertussis* under standard assay conditions. No significant difference in invasive capacity of organisms preincubated with anti-FHA at final concentrations of 0.25 and 0.50mg IgG/mL was demonstrated compared to untreated control organisms in the standard invasion assay (Table II.3). Preincubation of *B. parapertussis* 17903 with monovalent Fab fragments isolated from polyclonal antisera raised against this strain resulted in a marked decrease in invasion compared to untreated controls. A similar effect was observed with monovalent Fab fragments isolated from antisera raised against *B. pertussis* strain 2231 where invasion was reduced to a level of 27.6% of control values.

5. Demonstration of *B. parapertussis* invasion of human respiratory epithelium in primary culture.

Human respiratory epithelial cells were used as a more relevant cell line for testing the ability of *B. parapertussis* to invade human cells. Human respiratory epithelial cells were obtained from proteolytically disrupted nasopharyngeal turbinate tissue. Organisms were

added to confluent monolayers at initial MOI's of 0.1 and 1 and incubated for 24 hours. Viable count determinations following the 24 hours incubation period indicated an increase in MOI's to 100 and 1,000, respectively, corresponding to a division time of approximately two hours. Following extensive washing, intact monolayers were fixed *in situ* with 3.5% glutaraldehyde, processed and sectioned laterally from the apical face of the monolayer. TEM examination of ultrathin sections revealed moderate numbers of organisms within the cytoplasm of infected cells (Fig. II.4 A-C). Similar to those seen in sections of infected HeLa cells, intracellular organisms were bound by tight endocytic vacuoles.

D. DISCUSSION

In order to define the extent of *B. parapertussis* invasiveness, we compared it with several known invasive and noninvasive control bacterial species. *B. parapertussis* has a 3 hour lag phase and a lengthy generation time in MEM (2-3 hours). Thus, an initial ten-fold higher MOI was used for *B. parapertussis* than control organisms. Although this appears as an unfair advantage, the significantly shorter generation time of the control strains resulted in a dramatic increase in their numbers during the 5 hour incubation, resulting in final MOI's at

least five-fold greater than that of *B. parapertussis*. Final numbers of viable organisms remaining after gentamicin treatment reflect a combination of attachment, invasion, and intracellular multiplicaion potential of each strain; therefore, actual rates of invasion cannot be determined. *Bordetella sp.* attach efficiently to HeLa cell monolayers but have a limited capacity for intracellular growth during the assay period. In contrast, both *Salmonella hadar* and *Shigella flexneri* are capable of rapid intracellular multiplication once internalized, although binding efficiencies differ. When these differences are considered, the data indicate that, following a seven hour exposure period, approximately equal numbers of viable *B. parapertussis*, *Salmonella hadar* and *Shigella flexneri* exist within HeLa cells.

The intracellular existence of *B. parapertussis* was confirmed by TEM examination of infected monolayers. At intervals of 24, 48 and 72 hours following infection with *B. parapertussis* in the absence of gentamicin at an initial MOI of 0.1, monolayers were examined for intracellular organisms and relative changes in their numbers. Although intracellular numbers progressively increased, the process appeared limited as cells did not contain large numbers of intracellular organisms even at 72 hours post-infection. This is supported by viable count data which indicated that intracellular organisms did not replicate to any

appreciable extent. Consistent with these observations, most intracellular bacteria occured singly within endocytic vacuoles. Although organisms undergoing fission were occasionally observed, these may have been in the process of division during internalization.

The ability of invasive bacteria to self-regulate their intracellular numbers would lend the organisms a definite survival advantage. This is especially true for *B. parapertussis* which produces a number of potentially cytotoxic substances. Fewer intracellular organisms would minimize disruption of the host cell thereby providing shelter for the organisms from active nonspecific and specific immune response occuring in the extracellular environment surrounding the infected host cell. Alternatively, phase transition or phenotypic modulation to an avirulent phenotype as described for *B. pertussis* (7, 18) may follow internalization of *B. parapertussis* into an intracellular environment. Once established within the cytoplasm, a complete or partial loss of virulence-associated determinants would reduce cellular disruption caused by an intracellular organism, thereby potentiating its quiescent masquerade.

Bacterial "invasion" of non-professional phagocytes is likely accomplished by a phagocytic process (5). In this study we examined the mechanism of *B. parapertussis* invasion assuming that a similar process would be involved.

Receptor-mediated endocytosis (RME) is a common mechanism of internalization of several types of viruses which enter eucaryotic cells during normal RME-mediated uptake of physiologically important external proteins (11,12). We did not anticipate a role for RME in uptake of *B. parapertussis* due to the absence of characteristic clathrin seen in association with *B. parapertussis* endocytic vesicles as well as their relatively large size in comparison with clathrin-coated pits. Since the width of the bacteria is similar to that of chlamydia (0.3μm), it was possible that RME could have been involved in an "end-on" internalization mechanism similar to that pictured in Figure II.2C. To investigate this possiblity, we examined the effects of an RME inhibitor on uptake of *B. parapertussis*. Primary amines such as monodansylcadaverine (MDC) inhibit RME-mediated uptake of virus particles (14), presumably by inhibition of Ca^{++}-activated transglutaminases which normally stabilize protein ligand-receptor clustering in coated pits through covalent cross-bridge formation (4). As expected, pretreatment of HeLa cells with MDC had no effect on uptake of *B. parapertussis*, thereby suggesting that RME is probably not a significant route of entry. However, further experimentation with other inhibitors of RME, such as amantidine (14), could be performed to further discount this possiblity.

A microfilament-dependent endocytic process did appear to be involved, as adherent bacteria in the process of entry were usually seen circumscribed by outstretched microvilli. Pretreatment of HeLa monolayers with microfilament inhibitors cytochalasins B and D produced a marked inhibition of *B. parapertussis* uptake. This reduction was especially pronounced in the presence of cytochalasin D, the more potent microfilament inhibitor of the two cytochalasins tested. These data indicate that uptake of *B. parapertussis* proceeds by a phagocytic process, possibly as a result of a bacterium-host cell interaction which stimulates uptake of the former.

Recognition and adhesion are logical prerequisites to endocytosis by nonphagocytic eucaryotic cells. For example, the act of adhesion and/or the anchoring effect produced by adhesion of a bacterium to its corresponding cell-surface receptor could be an important stimulus which initiates catalysis or itself catalyzes endocytosis by the host cell. We attempted to inhibit endocytosis of *B. parapertussis* by pretreatment with hyperimmune sera raised against filamentous hemagglutinin (FHA), the putative major adhesin of *B. pertussis* (13, 16). *B. pertussis* and *B. parapertussis* possess forms of FHA which are similar in morphology, hemagglutinating ability and antigenic specificity. Thus, FHA is the presumed ligand of *B. parapertussis* even though the actual adhesin of this

species has never been formally identified. To the contrary, our data demonstrate that invasion is unhindered in the presence of anti-FHA. This suggests that adherence of these organisms is mediated by a ligand(s) other than FHA. This was supported by the observation that numerous adherent bacteria per HeLa cell were observed in Giemsa-stained monolayers infected with anti-FHA-treated organisms. These data may be especially significant with respect to acellular pertussis vaccines consisting of FHA and PT presently undergoing clinical trials. If currently used whole cell vaccines containing numerous antigens common to both pertussis and parapertussis fortuitously provide cross-protection against both species, as indicated by the ability of polyclonal anti-pertussis sera to reduce invasion of *B. parapertussis* shown here, exclusion of all antigens except FHA and PT which do not protect against *B. parapertussis* invasion may result in a much higher incidence of infection and/or disease caused by this organism.

HeLa and other continuous cell lines provide simple and convenient *in vitro* systems to investigate the processes of adherence and invasion, but these systems are quite different from the cell types and environment which pathogenic bacteria encounter following introduction into the host. Lack of cell differentiation, structural complexity, representative cell receptor types and numbers

and immune modulation are but a few examples of inherent differences between these systems which limit the pertinence of data obtained from continuous cell culture methods. Although difficult to closely simulate the milieu in which *B. parapertussis* becomes established in the human lung, it is possible to provide the bacteria with a stratum for invasion which more closely parallels that which is naturally encountered. In this study, human respiratory epithelial cells obtained from proteolytically disrupted nasopharyngeal turbinate tissues were used to represent cell types normally colonized by *B. parapertussis* in the infected host. Monolayers initially infected with *B. parapertussis* at MOI's of 0.1 and 1 were fixed *in situ* following a 24 hour coincubation period and examined by transmission electron microscopy. Ultrathin sections revealed clear evidence of bacterial invasion. Organisms were observed within the cytoplasm of infected cells, again bound by endocytic vacuoles whose limiting membranes appeared in close contact with the bacterial cell wall. Host cells and intracellular bacteria both appeared healthy; there was no evidence of death or disruption after the 24 hour coincubation period used here. These data support the invasive potential of *B. parapertussis* indicated in the HeLa cell system and extend it to a more relevant model which more closely simulates that which is naturally encountered by respiratory pathogens.

In summary, these data describe the unusual phenomenon of invasive behavior in a bacterial species that until now was strictly regarded as an extracellular pathogen. *Bordetella parapertussis* appears capable of provoking its uptake through endocytosis by nonphagocytic human cells. As entry without a means of intracellular survival would be suicidal, *B. parapertussis* must also possess mechanism(s) to either thwart rapid lysis by lysosomal contents following phagosome-lysosome fusion, prevent lysosomal fusion itself, or escape from the phagosome and replicate within the cytosol. Such mechanisms have not yet been defined. Clearly, adaptation to an intracellular existence is no simple task and must therefore offer a significant survival and/or pathogenic advantage to the species involved. *B. parapertussis* is no exception. Although perhaps not a pathogenic mechanism which contributes to tissue injury and histopathology, limited invasive behavior would lend a definite survival advantage to this species whose only host appears to possess a significant incidence of specific humoral immunity.

Table II.1

Comparison of Uptake of Invasive and Non-invasive Bacterial Strains by HeLa 229 Monolayers[a].

Strain	CFU/Monolayer x 10^5
Yersinia enterocolitica 0:3 E2549	490.7 ± 15.3
Yersinia pseudotuberculosis Type 1A	89.8 ± 9.7
Salmonella hadar	25.9 ± 6.1
Shigella flexneri[b]	14.8 ± 0.3
Bordetella parapertussis 17903	24.3 ± 2.3
Escherichia coli 10418[c]	1.1 ± 0.8
Escherichia coli 10407P[d]	0.3 ± 0.1
Escherichia coli SA 1377[e]	1.2 ± 0.7

a. HeLa 229 cell monolayers (ca. 1.5 x 10^6 cells per 25-cm^2 flask) were infected with 1.5 x 10^8 (*B. parapertussis*) or 1.5 x 10^7 (all other strains) organisms. Data represent the mean number of CFU per monolayers ± the SD of triplicate independent determinations.
b. Strain contains the 140-megadalton plasmid and the lipopolysaccharide O side chain required for virulence.
c. Highly piliated strain.
d. Nonpiliated variant.
e. Rough lipopolysaccharide mutant, chemotype Re.

Table II.2

Effect of Cytochalasins B and D on Internalization of *B. parapertussis* 17903 by HeLa 229 Monolayers.[a]

Cytochalasin (μg/mL)		Organisms per Monolayer, % of Control
Cytochalasin B	1.0	17.6 ± 5.2
	2.5	16.2 ± 7.7
Cytochalasin D	1.0	1.0 ± 0.4
	2.5	0.7 ± 0.4

a. Data are expressed as the mean ± SD of 4 replicates for cytochalasin B and 6 replicates for cytochalasin D determinations.

Table II.3

Effect of antisera on internalization of *B. parapertussis* by HeLa 229 cells.[a]

Antisera	Protein Concentration (mg/mL)	Organisms per Monolayer % of Control
Rabbit anti-*B. parapertussis* 17903	0.25	16.4 ± 3.6
Rabbit anti-*B. pertussis* 2231	0.25	27.6 ± 8.4
Goat anti-*B. pertussis* filamentous hemagglutinin (FHA)	0.25	101.0 ± 7.1
	0.50	104.4 ± 5.2

a. Results represent average number of organisms per monolayer expressed as percentage of control ± SD, n=6 for anti-*B. parapertussis*, n=7 for anti-*B. pertussis* and n=4 for anti-FHA data.

FIGURE II.1

Transmission electron micrographs of intracellular *B. parapertussis* 17903 within HeLa 229 monolayers. Panels (A) and (B) represent monolayers sectioned 48 hours post-infection, (C) 72 hours post-infection. Intracellular bacteria are seen bound by tight endocytic vacuoles.

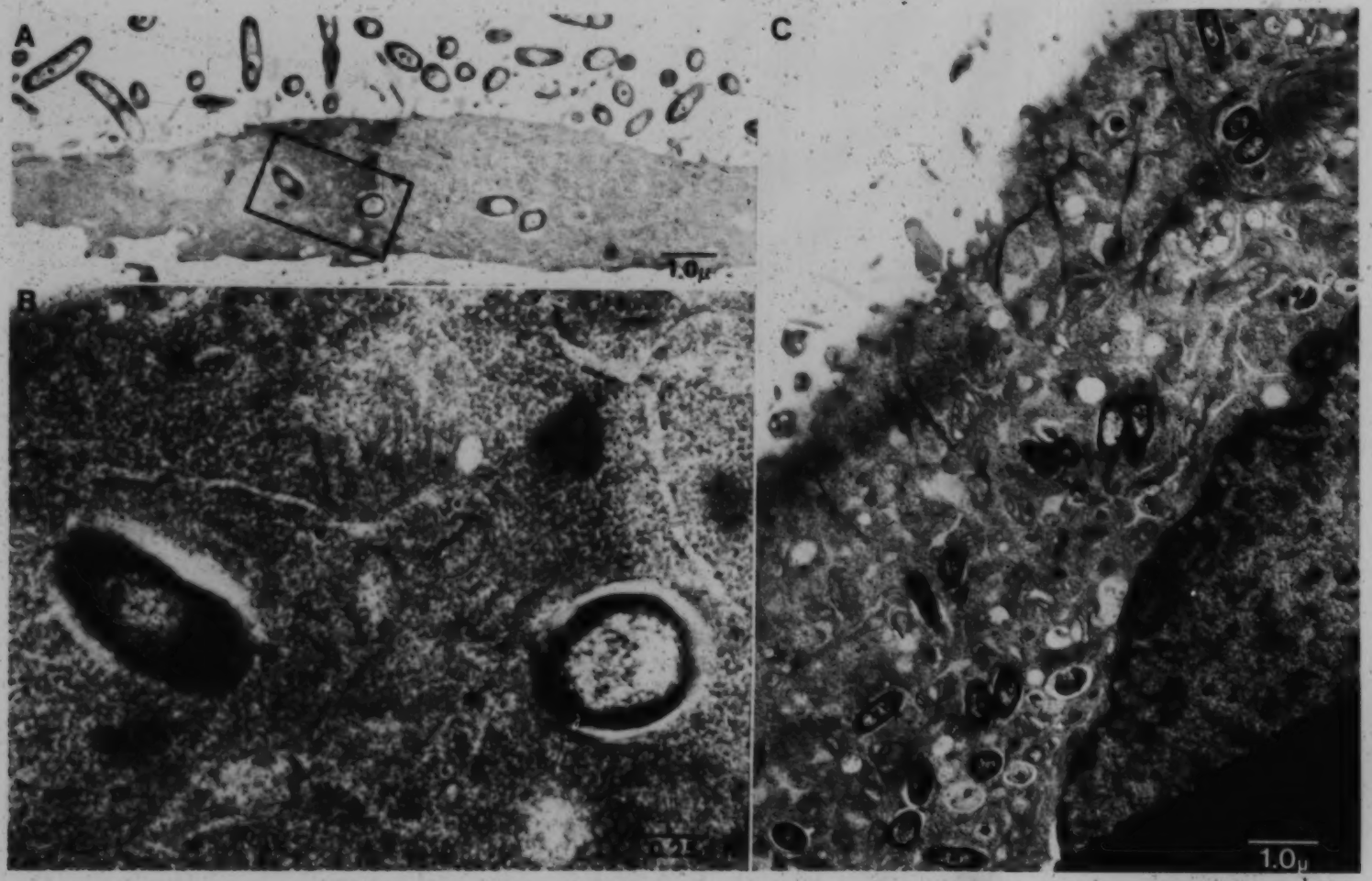
A
1.0μ
B
C
1.0μ

FIGURE II-2

Transmission electron micrographs of HeLa 229 monolayers infected with *B. parapertussis* 17903. Panels (A)-(C) demonstrate bacteria in various stages of internalization.

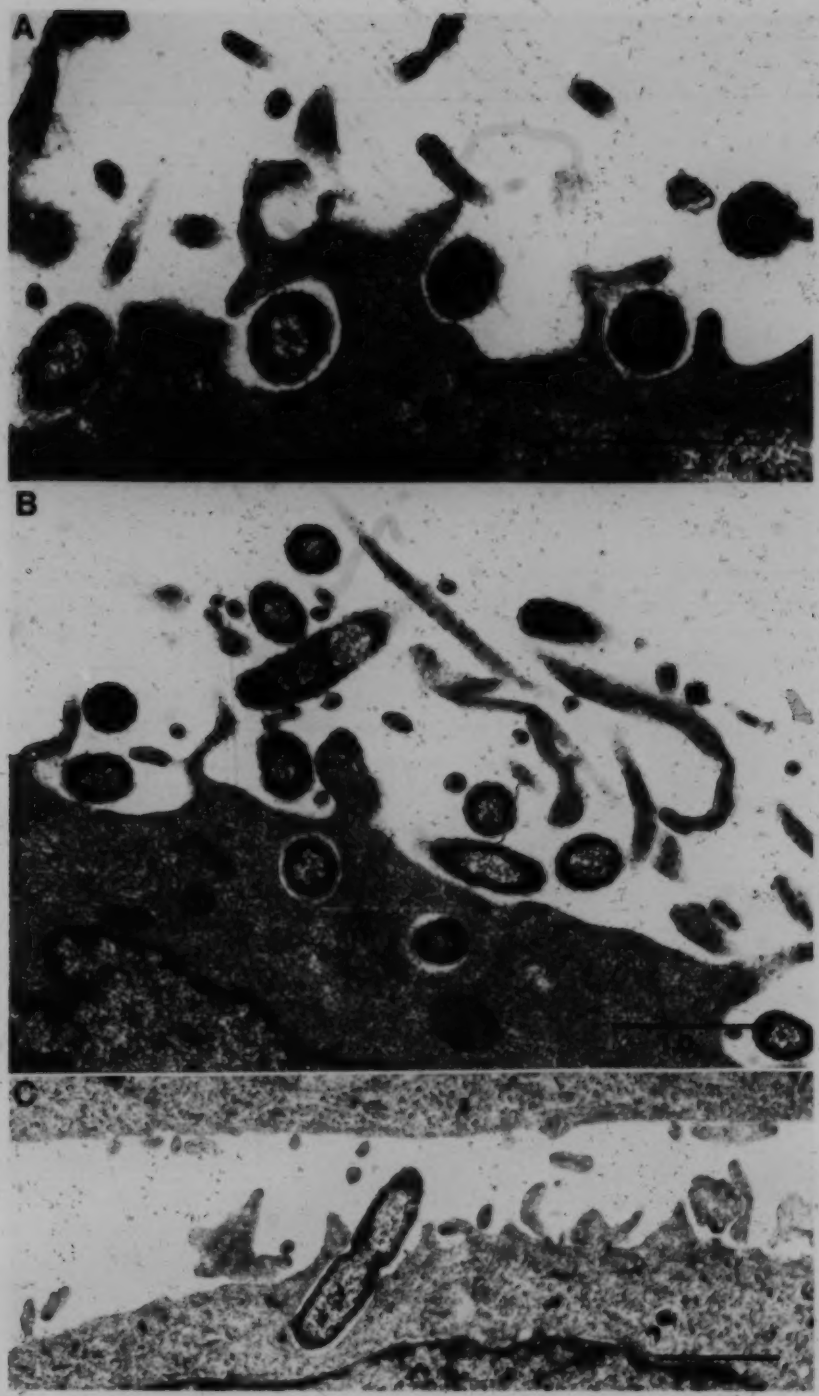
A
B
C

FIGURE II.3

Recognition of *B. parapertussis* 17903 FHA by purified IgG antibodies raised against anti-*B. pertussis* FHA demonstated by immunoblotting. Whole cell lysates of Bordetellae were electrophoresed through a 10% denaturing SDS-PAGE, transferred to nitrocellulose and probed with goat anti-FHA. The molecular weight standards are marked (in thousands) along the side of the immunoblots.

FIGURE 11.3

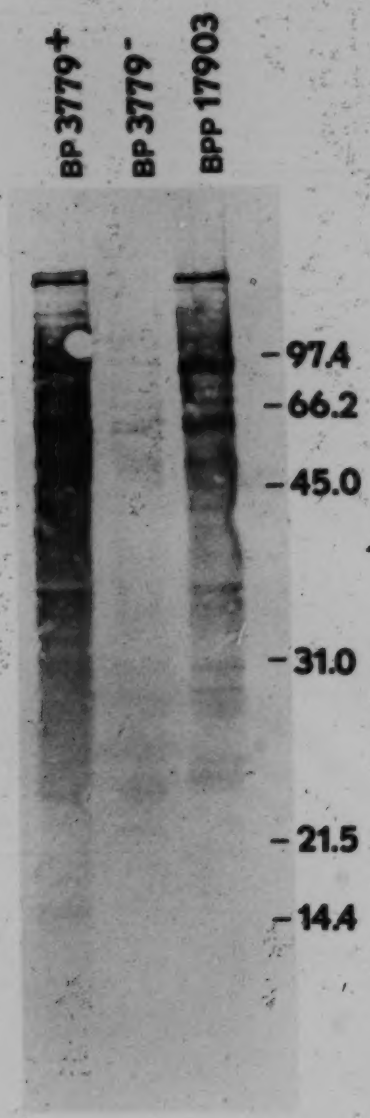

FIGURE II.4

Transmission electron micrograph of human respiratory epithelial cells in primary culture infected with *B. parapertussis* 17903. Arrows indicate intracellular bacteria bound by tight endocytic vacuoles.

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Chapter III

INVASION OF HELA 229 CELLS BY VIRULENT *BORDETELLA PERTUSSIS*[1]

A. INTRODUCTION

Bordetella pertussis is generally thought to be a noninvasive organism which causes disease in its human host through the action of several potent toxins. Classical whooping cough is believed to occur in two main stages (15). Initally, inspired bacilli colonize epithelial surfaces in the upper respiratory tract. Candidate adhesins include filamentous hemagglutinin (19,20), pertussis toxin (19), fimbriae (8,16) and pertactin (Leininger *et al.*, in press) Once established, pathogenesis is caused by a number of toxins produced by *B. pertussis*. Local cytotoxic effects (7) and systemic metabolic disturbances (6,13,14), combine to produce the characteristic symptoms of a disease which is primarily regarded as a toxicosis (15, 17).

Having explored the ability of *B. parapertussis* to invade cultured eucaryotic cells in the previous chapter, the following chapter continues along the same vein as the invasive capacity of *B. pertussis* is characterized. Since the incidence of whooping cough caused by *B. pertussis* is

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[1] A version of this chapter has been published. **Ewanowich, C. A., A. R. Melton, A. A. Weiss, R. K. Sherburne, and M. S. Peppler.** 1989. Infect. Immun. 57:2698-2704.

significantly greater than that caused by *B. parapertussis*, significantly more research effort has been devoted toward characterization of the pathogenic mechanisms of the former species. This worked to our advantage when we began to examine the invasive capacity of *B. pertussis*, as a number of mutants containing Tn*5* or Tn*5 lac* inserts in various virulence determinants were already available for our purposes (21,22). The use of these mutants allowed us to dissect the relative contributions of various virulence-associated gene products to *B. pertussis* invasion, as described in this chapter.

B. MATERIALS and METHODS

1. Bacterial Strains.

Transposon insertion derivatives of a nalidixic acid resistant strain of *Bordetella pertussis* Tohama 1 (BP338) used in this study are listed in Table III.1. Tn*5* or Tn*5 lac* insertions in various virulence regulated genes (denoted by prefixes BP or BPM, respectively) were constructed as described previously (21,22). *Shigella*, *Salmonella*, and *Yersinia* strains were previously described (4).

2. Invasion Assays.

HeLa 229 cells (human epithelium-like, ATCC CCL 2.1) were used in invasion assays as previously described (4)

with the following modifications. Twenty-four well tissue culture trays were seeded with approximately 7×10^4 cells per well 18 hours prior to assay. Cultures of *B. pertussis* grown 48 hours on BGA were suspended in Eagle's Minimal Essential Medium (MEM) (Gibco, Grand Island, New York) supplemented with 3% Fetal Bovine Serum (FBS) (Flow Laboratories, McLean, Va.) to an optical density of 0.12 in 13 x 100mm tubes (pathwidth = 1.0cm) at 540nm. Approximately 9×10^6 colony-forming units (CFU) in 0.4mL were added to each well and incubated at 37°C, 5% CO_2 under static conditions for 5 hours, during which time little or no multiplication of *B. pertussis* occurred due to the organisms' relatively lengthy generation time and initial lag phase upon addition to the monolayers. Monolayers were then washed twice to remove nonadherent bacteria and the medium was replaced with 1.0mL of MEM/FBS containing 100µg/mL gentamicin (Gibco) and incubated at 37°C for 2 hours to destroy the remaining extracellular bacteria. After 2 hours, residual gentamicin was removed by extensive washing, and monolayers were harvested for quantitation of intracellular CFU's. In separate experiments, treatment of *B. pertussis* strains suspended to a density equivalent to that used in the invasion assays with 100µg/mL gentamicin for 2 hours resulted in a 99.998% decrease in the number of viable bacteria.

3. Alteration of HeLa cells.

In several experiments, HeLa monolayers (approx. 7 x 10^4 cells) were treated with individual reagents for 1 hour at 37°C prior to addition of bacteria as indicated above. Cholera toxin (List Biological Laboratories, Campbell, California) was used at a final concentration of 30ng/mL. Forskolin (Sigma Chemical Co. St. Louis, Missouri) was dissolved in 95% ethanol and used in assays at 100µM concentration. Cytochalasin D (Sigma) was used as previously described (4, Chapter 2). Due to the reversibility of the actions of cytochalasin D and forskolin, the concentrations of all reagents were maintained throughout the duration of each assay.

4. Pretreatment of *B. pertussis* with Filamentous Hemagglutinin.

Purified filamentous hemagglutinin (FHA) was kindly provided by John Vose and Larry Tan of Connaught Laboratories, Willowdale, Ontario. *B. pertussis* strains grown 48 hours on BGA were suspended in Hanks balanced salt solution (HBSS) containing 25mM HEPES (*N*-[2-hydroxethyl]piperazine-*N*'-2-ethanesulfonic acid) (pH 7.2) (HBSS-HEPES) to an optical density of 0.12 at 540nm. FHA in 54mM NaH_2PO_4-13mM K_2PO_4-1M NaCl pH 8.0 was added to final concentrations of 25, 50, and 75µg/mL. Control organisms received no additions. Suspensions were incubated under static conditions for 1 hour at 37°C, washed once,

resuspended in MEM to the original volume, and used in invasion assays as described above.

5. Effect of Antibody on Internalization.

Purified IgG fraction of goat anti-FHA was kindly provided by Drs. Jim Cowell and Michael Brennan, Division of Bacterial Products, Office of Biologics and Research and Review, Center for Drugs and Biologics, Food and Drug Administration, Bethesda, Maryland. Monovalent Fab fragments purified from polyclonal antisera were used as described previously (4).

Monoclonal antibodies purified from ascities fluid directed against *B. pertussis* agglutinogens, lipo-oligosaccharide A and pertactin, a non-fimbrial 69 Kd surface antigen of BP338 (serotype 1.2.3.4) (1) were kindly provided by Dr. Michael Brennan. Bacteria resuspended in HBSS-HEPES as described above were preincubated with antisera at a final protein concentration of 50µg/mL for 1 hour at 37°C under static conditions, diluted and used in standard invasion assays.

6. Cyclic AMP Quantitation.

Cyclic AMP levels in control and treated HeLa cells were determined 6 hours after addition of cholera toxin (30ng) or forskolin (100µM). This interval corresponds to the total time which monolayers are exposed to these reagents in invasion assays. 3-isobutyl-1-methylxanthine

(IBMX, Sigma Chemical Co.) at a final concentration of 0.5mM was present throughout the assays in order to minimize destruction of cyclic AMP by cellular phosphodiesterases. HeLa cells were scraped from flasks with a rubber policeman, homogenized and boiled for 3 minutes to deproteinize the samples and release cyclic AMP. Available cyclic AMP was quantitated using an assay based on competition for a binding protein with added ^{3}H-cyclic AMP (Amersham Canada Limited, Oakville, Ontario).

7. Transmission Electron Microscopy.

Processing and examination of infected HeLa monolayers by transmission electron microscopy was performed by Richard K. Sherburne, Department of Medical Microbiology and Infectious Diseases, University of Alberta, Edmonton, Alberta, Canada. HeLa monolayers (approx. 7×10^4 cells) were grown for 24 hours in 24-well tissue culture trays lined with glass coverslips. Approximately 9×10^6 *B. pertussis* from 48 hour BGA cultures were added to each monolayer and coincubated for 7 hours at 37°C under static conditions. At 7 hours post-infection, monolayers were washed and processed for TEM examination as previously described (4).

C. RESULTS

1. Virulence-Dependent Uptake of *B. pertussis* by HeLa Cells.

We first compared numbers of viable intracellular phase 1 *B. pertussis* recovered from infected monolayers treated with gentamicin to those of known invasive and non-invasive control strains after a 7 hour coincubation period (Figure III,1). The invasion process is time-dependent and theoretically a shorter interval could have been used. However, in our adherence assays, the number of bound organisms requires 3 hours to reach equilibrium. Thus, we empirically decided on a 5 hour invasion period to allow for maximal adherence and an additional 2 hours for invasion. This interval could have been shortened by prior centrifugation of the bacteria onto the monolayers, but we did not wish to introduce potential artifacts to further confuse the interpretation of the data. After the 7 hour invasion interval, numbers of viable intracellular BP338 were comparable to those of invasive clinical isolates of *Shigella flexneri* and *Salmonella hadar*. Expression of virulence determinants in *B. pertussis* is coordinately regulated by an activator encoded by a gene known as *vir*. Mutations within this gene abolish expression of all virulence factors encoded by *vir*-regulated genes. Recovery of avirulent Tn*5*-insertion mutant BP347 was comparable to that of noninvasive *Escherichia coli* strains, indicating

that invasion by *B. pertussis* is dependent on the expression of one or more *vir*-regulated genes. In order to determine which of the numerous *vir*-regulated genes of *B. pertussis* were required for invasion, we compared the relative invasiveness of a number of Tn*5* and Tn*5 lac* insertion mutants derived from parental strain BP338 (Figure III.1). Each of these mutants lacks one or more virulence-associated proteins, some of which have not yet been characterized.

Two virulence determinants appear to be required for maximal internalization. The invasion levels of *B. pertussis* strains which lack only filamentous hemagglutinin (FHA) (BP353, BPM409 and BPM1821) were less than that of BP338 (FHA^+) but significantly higher than avirulent BP347, also devoid of FHA. The Tn*5* mutant BP357 deficient in functional pertussis toxin production also demonstrated a significant reduction in invasion compared to BP338. Invasion of BPM3171, which produces functional pertussis toxin despite a transposon insertion into a pertussis toxin-linked gene, was not significantly reduced compared to the wild type strain.

Another factor was found to influence invasion in an unexpected way. Surprisingly, invasion of *B. pertussis* strains lacking expression of the bifunctional protein which specifies adenylate cyclase toxin activity and hemolytic activity (BP348, BP349, BPM433 and BPM3183) exceeded that of parental BP338 by up to two-fold.

Two mutants with Tn5 *lac* insertions in undefined *vir*-activated genes (BPM2119 and BPM2041) also demonstrated significant reductions in invasion compared to wild type ($P < 0.05$; approximate t-test used to determine P values due to large range in variances which existed among strains tested [18]). Adherence of these strains to HeLa cells is not significantly reduced compared to wild type. Similarly, ^{125}I-labelled surface exposed protein profiles of these strains are identical to BP338 (data not shown). This indicates that these strains may lack expression of a protein required for invasion itself or subsequent intracellular survival.

2. Effect of Cyclic AMP on Invasion.

The enhanced invasiveness of *B. pertussis* mutants lacking adenylate cyclase toxin led us to question the influence of cyclic AMP on invasion of *B. pertussis*. Intracellular levels of cyclic AMP in HeLa cells were increased by prior incubation with either cholera toxin or forskolin. The treated cells were then infected with *B. pertussis* mutants lacking adenylate cyclase toxin and hemolysin to determine the effect of elevated intracellular cAMP on invasion of these strains. Initial results indicated that the level of cyclic AMP in HeLa cells exposed to cholera toxin or forskolin was below the sensitivity of the assay employed (<2.0 pmol/10^7 cells). To confirm that these agents caused a relative increase in

cyclic AMP in HeLa cells exposed to these agents compared with controls, we included 0.5mM 1-isobutyl-3-methylxanthine (IBMX) in later assays in order to prevent destruction of newly generated cyclic AMP by phosphodiesterases. IBMX was not included with cells subsequently infected with *B. pertussis*. Table III.2 indicates that forskolin and cholera toxin cause a detectable increase in intracellular cyclic AMP in HeLa cells. Furthermore, the level of invasion of each mutant lacking adenylate cyclase toxin and hemolysin was reduced to a level similar to the parental strain in cells exposed to either cholera toxin or forskolin (Table III.3). These data indicate that elevated levels of cAMP produce an inhibition of invasion of *B. pertussis*. Invasion of both BP338 and BP347 were unaffected by increases in cyclic AMP.

As a correlative experiment, we subjected treated monolayers to invasion by an unrelated species, *Yersinia pseudotuberculosis* Type 1A. Invasion by *Y. pseudotuberculosis* was reduced to levels of 38.0 ± 9.1 % and 34.9 ± 2.0 % (n=2) of untreated controls in the presence of cholera toxin and forskolin, respectively. These data suggest that increases in cyclic AMP cause a generalized reduction in phagocytic activity of the HeLa cells.

3. Effect of Exogenous FHA on Invasion of *B. pertussis* FHA⁻ Mutants.

The invasion data indicated that the presence of FHA was necessary for optimal invasion of *B. pertussis*. We preincubated BP353 (FHA⁻) with purified FHA prior to invasion assays to determine whether exogenously-supplied FHA would enhance invasion of these strains. The data shown in Figure III.2 indicate an increase in invasion of BP353 preincubated with concentrations of FHA ranging from 0-75 μg/mL.

4. TEM Examination of HeLa Monolayers Infected with *B. pertussis*.

Sparsely-seeded HeLa monolayers on glass coverslips initially infected with approximately 9×10^6 BP338 or BP348 for 7 hours were examined by TEM in order to confirm the intracellular location of these organisms. Strain BP348 lacking both adenylate cyclase toxin and hemolysin was included to determine if this more invasive mutant would produce a morphological change in the intracellular appearance of these organisms. In Figure III.3, panels A-D depict parental strain BP338 within the cytoplasm of infected HeLa cells. In the majority of cases, bacteria occurred singly within phagosomes, suggesting that intracellular multiplication does not occur within the 7 hour interval. Phagosomal membranes surrounding the bacteria appeared to be in close contact with the

circumference of the bacterial cell wall, often hindering their recognition. The intracellular appearance of mutant BP348 in Figure III.3, panel F, was not appreciably different from that of BP338. In some instances, bacteria were observed in the process of internalization, partially or completely enveloped by microvilli. An example of this process is seen in Figure III.3, panel E.

5. Entry of *B. pertussis* via a Microfilament-Dependent Process.

TEM observations suggested that *B. pertussis* enters HeLa cells by a phagocytic process. Since phagocytosis requires the action of microfilaments proximal to the site of entry, HeLa cells were pretreated with cytochalasin D, a potent microfilament inhibitor, to determine if it had an effect on *B. pertussis* uptake. In the presence of 1.0 and 2.5 µg/mL cytochalasin D, invasion was reduced to 1.7 ± 0.4 and 1.4 ± 0.5% of untreated controls, respectively (mean ± SD, n=9). Thus cytochalasin D lowered levels of invasion to that of avirulent strain 347 and suggests that invasion of *B. pertussis* proceeds via a microfilament-dependent phagocytic-like process. The presence of cytochalasin B or D did not reduce the viability of the bacteria, however.

6. Effect of Monoclonal and Polyclonal Antisera on Invasion of *B. pertussis*.

We pretreated BP338 (serotype 1.2.3.4.) with various purified monoclonal and polyclonal antibodies in order to more clearly define the role of specific antigens in invasion by *B. pertussis*. The results are listed in Table III.4. Preincubation with antisera raised in rabbits against *B. parapertussis* 17903 did not reduce invasion of BP338. Pretreatment with rabbit anti-*B. pertussis* 2231, however, resulted in a dramatic reduction in invasion to a level of 4.5 ± 2.1% of control. Based on our results with FHA⁻ Tn*5* *lac* mutants, we expected that pretreatment of wild type *B. pertussis* (BP338) with anti-FHA would inhibit invasion. We used an extant anti-FHA preparation under identical conditions in which it has been proven to inhibit binding of *B. pertussis* Tohama phase 1 to WiDr cells (24). Indeed, a reduction in invasion similar in magnitude to the reduction in binding to WiDr cells reported by Urisu et. al (24) was observed following pretreatment of BP338 with 0.25 and 0.50 mg/mL anti-FHA. FHA appears to play a pivotal role in invasion of HeLa cells by *B. pertussis*, likely through adhesion to the eucaryotic cell membrane.

Invasion of BP338 was also tested after pretreatment with a number of monoclonal antisera raised to various surface antigens (Table III.4). Monoclonal antisera to fimbrial antigens 2 or 3/6 had no effect on invasion. Three monoclonal antisera which recognize a newly-described

69 kDa surface protein (1) did not reduce invasion of BP338. One of the monoclonal antibodies (BPE3) recognizes a potentially adhesive epitope, since it has been reported to reduce adherence of CHO cells to the 69 kDa protein (E. Lieninger-Zapata, M. J. Brennan, J. G. Kenimer, I. Charles, N. Fairweather, and P. Novotny. 1989. Abstr. Ann. Meet. Am. Soc. Microb. B123, p.51). In contrast, a significant reduction in invasion resulted from treatment of BP338 with a monoclonal antibody directed against lipooligosaccharide A.

D. DISCUSSION

This chapter demonstrates the invasive behaviour of *B. pertussis*, commonly regarded as a noninvasive respiratory pathogen. Virulent phase *B. pertussis* invade HeLa cells, whereas avirulent organisms do not, indicating the dependence of this behavior on *vir*-regulation. Mutants lacking FHA showed significantly decreased invasion, presumably by a reduction in adherence. Accordingly, preincubation of FHA-deficient strains with exogenous FHA resulted in a dose-dependent increase in uptake. While there exists strong evidence to suggest that FHA mediates adherence of *B. pertussis* to several cell types *in vitro*, (16, 19, 20) it is important to note that its role *in vivo* is still unclear. It is believed to mediate adherence of *B. pertussis* to ciliated cells of the upper respiratory tract,

but its role in the interaction of *B. pertussis* with nonciliated cell types of the respiratory tract is unknown.

Expression of pertussis toxin also appears to contribute to invasion. Previous data have indicated that pertussis toxin, in combination with FHA, mediates binding of *B. pertussis* to human ciliated respiratory cells (20), and Vero cells (8). This suggests that the reduced invasion of the pertussis toxin mutant may be due to a reduction in adherence. However, the possibility that pertussis toxin may mediate intracellular survival of *B. pertussis* cannot be excluded.

Of interest is the unusual level of invasion demonstrated by the adenylate cyclase toxin/hemolysin mutants BP348, BP349, BPM433 and BPM3183. Intoxication of professional phagocytes by adenylate cyclase toxin inhibits phagocytic and oxidative responses via increases in cyclic AMP. (3, 7, 13, P. Symes, E. L. Hewlett, D. Roberts, A. Q. de Sousa and R. D. Pearson. 1983. Clin. Res. p. 377A). In addition, increases in cyclic AMP have also been shown to inhibit phagocytic activities of polymorphonuclear leukocytes (3). However, the specific effect of cyclic AMP increases on parasite-directed phagocytosis by nonprofessional phagocytes is unknown. Our data suggest that increases in cyclic AMP, generated either by strains of *B. pertussis* which produce adenylate cyclase toxin, or by addition of exogenous agents, attenuates the phagocytic activity of HeLa cells. Conversely, the uptake of strains

lacking adenylate cyclase toxin is increased, and, accordingly, reconstitution of increased levels of cyclic AMP restores the attenuation and results in a reduction in phagocytosis. A similar effect on Shigella invasion was previously reported by Hale *et al.* (9). Increased cAMP levels in Henle 407 monolayers following treatment with either dibutryl cyclic AMP or cholera toxin caused a significant reduction in invasion by these organisms (9). Inhibition of phagocytosis by adenylate cyclase toxin may represent a mechanism by which the bacteria limit their own uptake to minimize disruption of the host cell caused by the numerous cytotoxic substances they produce. Further experimentation is needed to determine the mechanism by which *B. pertussis* increases cyclic AMP in HeLa cells. Such studies may also reveal other effects of increased cyclic AMP on recovery of viable counts of invasive bacteria.

Invasion was markedly reduced by prior incubation of the bacteria with monovalent Fab fragments of polyclonal whole cell anti-pertussis antibodies. In contrast, Fab fragments of polyclonal anti-parapertussis antibodies had little effect. This is important because both anti-pertussis and anti-parapertussis significantly reduced uptake of *B. parapertussis*, as shown in the previous chapter (Table II.3). These data suggest that *B. pertussis* possesses unique antigenic determinants absent on *B. parapertussis* which are important for adherence and/or

invasion. Consistent with our results is a report from Kendrick *et al.* which demonstrated that intraperitoneal immunization with *B. parapertussis* does not protect mice against intracerebral challenge with *B. pertussis* (11).

Preincubation of the organisms with polyclonal IgG anti-FHA showed a reduction in invasion similar in magnitude to the reduction in adherence previously described for *B. pertussis* Tohama 1 under identical conditions (20). Interestingly, the same antiserum had no effect on invasion of HeLa cells by *B. parapertussis* (4), although the FHA's of both species are presumed to be similar. These data indicate that important antigenic differences exist between FHA's of *B. pertussis* and *B. parapertussis*.

Anti-lipooligosaccharide A (LOS A) caused a significant reduction in uptake. Recent data suggest that LOS A may constitute part of agglutinogen 1 of *B. pertussis* (12). This is relevant because a monoclonal antiserum raised against agglutinogen 1 has previously been shown to reduce adherence of *B. pertussis* to Vero cells (8). In view of these data, the reduction in invasion caused by anti-LOS A may simply reflect a reduction in adherence. Alternately, the presence of a large number of antibodies on the surface of *B. pertussis* may impair an interaction between relevant surface adhesins and the HeLa cell surface. In this case, the reduction in invasion would be a secondary effect due to steric hindrance.

The intracellular presence of *B. pertussis* was confirmed by TEM examination of infected monolayers. The lack of an apparent vacuole enclosing each bacterium frequently complicated recognition of intracellular bacteria. The difficulty in recognizing these bacteria suggests an explanation for why intracellular *B. pertussis* have not been previously observed.

Induction of uptake by a non-phagocytic cell type and adaptation to a hostile intracellular environment are difficult tasks undertaken by only a few bacterial species. This suggests that an intracellular location confers a significant pathogenic and/or survival advantage to the species which develop these abilities. In the case of *B. pertussis*, intracellular survival would provide effective protection against both non-specific and specific host defenses, including humoral immunity. The latter is especially important considering the high incidence of immunity to pertussis produced by immunization. As such, prolonged intracellular carriage of *B. pertussis* in asymptomatic individuals may represent a sophisticated reservoir for transmission of pertussis. Further experimentation with respect to the mechanism of survival of intracellular *B. pertussis*, the possible occurrence of antigenic modulation within an intracellular environment containing appropriate signals, and demonstration of the occurrence of this phenomenon *in vivo* in relevant animal models is clearly indicated.

TABLE III.1

List of Strains Used in this Study[a]

Strain (genotype)	ACT	Hly	FHA	DNT	69kDa	PTX	Reference
BP338 (parental)	+	+	+	+	+	+	21
BPM1579 (unknown)[b]	+	+	+	+	+	+	22
BPM2123 (unknown)	+	+	+	+	+	+	22
BPM2859 (unknown)	+	+	+	+	+	+	22
BPM177 (unknown)	+	+	+	+	+	+	22
BPM245 (unknown)	+	+	+	+	+	+	22
BPM2055 (unknown)	+	+	+	+	+	+	22
BPM2119 (unknown)	+	+	+	+	+	+	22
BPM2041 (unknown)	+	+	+	+	+	+	22
BPM3171 (unknown)[c]	+	+	+	+	+	+	22
BPM2375 (pleiotropic)	±	±	±	±	±	±	22
BPM433 (*adc-1*::Tn*5 lac*)	-	-	+	+	+	+	22
BP349 (*hly-2*::Tn*5*)	-	-	+	+	+	+	21
BP348 (*hly-1*::Tn*5*)	-	-	+	+	+	+	21
BPM3183 (*adc-2*::Tn*5 lac*)	-	-	+	+	+	+	22
BPM1809 (*dnt-1*::Tn*5 lac*)	+	+	+	-	+	+	22
BP357 (*ptx-2*::Tn*5*)	+	+	+	+	+	-	21
BPM1821(*fhaA-1*::Tn*5 lac*)	+	+	-	+	+	+	22
BPM409(*fhaB-1*::Tn*5 lac*)	+	+	-	+	+	+	22
BP353 (*fha-1*::Tn*5*)	+	+	-	+	+	+	21
BP347 (*vir-1*::Tn*5*)	-	-	-	-	-	-	21
Salmonella hadar[d]							4
Shigella flexneri serotype 2[e]							4
Escherichia coli 10418 (P+)							4

a. Abbreviations: ACT, adenylate cyclase toxin activity; Hly, hemolysin activity; FHA, filamentous hemagglutinin determined by hemagglutination and immunoblotting, DNT, dermonecrotic toxin activity assayed in infant mice; 69-kDa protein, *vir*-specific non-fimbrial 69-kDa outer membrane protein; PTX, pertussis toxin activity.

b. Tn*5 lac* mutants with an unknown genotype lacked production of uncharacterized protein whose expression was virulence regulated.

c. The strain contained a Tn*5 lac* insertion in a pertussis toxin-linked gene; it expressed functional pertussis toxin.

d. Clinical isolate, smooth lipopolysaccharide.

e. Clinical isolate with 140-MDa plasmid and lipopolysaccharide O side chain requried for virulence.

Table III.2.

Stimulation of Cyclic AMP Levels in IBMX-treated HeLa 229 Monolayers by Cholera Toxin and Forskolin.

	pmol cyclic AMP/10^7 HeLa 229 cells[a]
Control HeLa	78.0 ± 2.0 (n=3)
HeLa + cholera toxin (30 ng/mL)	152.8 ± 51.3 (n=5)
HeLa + forskolin (100 µM)	214.0 ± 72.7 (n=5)

a. Cyclic AMP levels of HeLa monolayers (ca. 10^7 cells) were quantitated following an 8 hour coincubation period with 0.5mM 1-isobutyl-3-methylxanthine (IBMX) ± cholera toxin (30 ng/mL) or forskolin (100 µM) at 37°C. Concentrations of each were maintained throughout the duration of each experiment.

Table III.3.

Effect of Cholera Toxin and Forskolin on Invasion of HeLa 229 cells by *B. pertussis* Adenylate Cyclase Toxin Mutants

Strain (Phenotype)	Effect of[a]		
	Control	Cholera Toxin (30ng/mL)[b]	Forskolin (100μM)
BP338 (parental)	109.7 ± 25.5	98.2 ± 12.4	117.4 ± 34.9
BPM433 (ACT^-Hly^-)	212.5 ± 32.5	94.5 ± 10.5	133.2 ± 17.8
BPM3183 (ACT^-Hly^-)	173.1 ± 28.8	102.8 ± 5.2	101.9 ± 14.6
BPM348 (ACT^-Hly^-)	213.2 ± 12.0	97.3 ± 17.1	113.1 ± 4.1
BPM349 (ACT^-Hly^-)	194.1 ± 11.3	108.5 ± 10.4	118.4 ± 7.5
BP347 (Vir^-)	2.2 ± 1.0	1.4 ± 0.6	1.6 ± 1.1

a. Data represent the mean ± standard deviation CFU per monolayer (in thousands) for three independent determinations. Invasion of each of the ACT mutants was significantly reduced ($P<0.005$) in the presence of either cholera toxin or forskolin.

b. Cholera toxin and forskolin were preincubated with monlayers for 1 hour prior to the addition of *B. pertussis* in order to stimulate an increase in cyclic AMP. Due to the reversible effect of forskolin, the indicated concentrations were maintained throughout the experiment.

Table III.4.

Effect of Monoclonal and Polyclonal Antisera on Internalization of *B. pertussis* 338 by HeLa 229 cells

Antisera and specificity of antiserum	Protein concentration (mg/mL)	Organisms/ Monolayer (% of control)[a]
Polyclonal Antisera		
Rabbit anti-*B. parapertussis* 17903[b]	0.25	96.3 ± 26.5
Rabbit anti-*B. pertussis* 2231[b]	0.25	4.5 ± 2.1
Goat anti-*B. pertussis* filamentous hemagglutinin[c]	0.25	40.3 ± 6.0
	0.50	27.6 ± 4.5
Monoclonal Antisera[d]		
BPG10 Anti-LOS[e] A	0.05	41.5 ± 13.6
BPE8 Anti-69K	0.05	86.1 ± 8.2
BPE3 Anti-69K	0.05	91.4 ± 15.8
BPD8 Anti-69K	0.05	99.8 ± 1.4
BPF2 Anti-Fimbriae 2	0.05	110.3 ± 13.0
BPC10 Anti Fimbriae 3/6	0.05	101.7 ± 12.7

a. Results represent average number of CFU's per monolayer expressed as a percentage of control ± SD, n=6 for anti-*B. pertussis* and anti-B. *parapertussis* data, n=9 for anti-FHA data.
b. Monovalent Fab fragments were isolated following papain digestion of IgG eluate from Protein A-Sepharose CL-4B column.
c. Purified IgG fraction of goat anti-FHA.
d. Monoclonal antisera were raised against *B. pertussis* serotype 1.2.3.4). Data represent mean ± SD of 3 independent determinations for each monoclonal antiserum, except BPG10 where n=9.
e. LOS, lipooligosaccharide.

FIGURE III.1

Invasion of HeLa 229 monolayers by *B. pertussis* mutants. Values represent 1000's of CFU recovered from gentamicin-treated monolayers, mean ± SD for 6 replicates. *, $P < 0.05$, §, $P < 0.1$ compared to wild type.

Invasion of HeLa 229 Monolayers by *B. pertussis* 338 Tn*5* and Tn*5 lac* Mutants

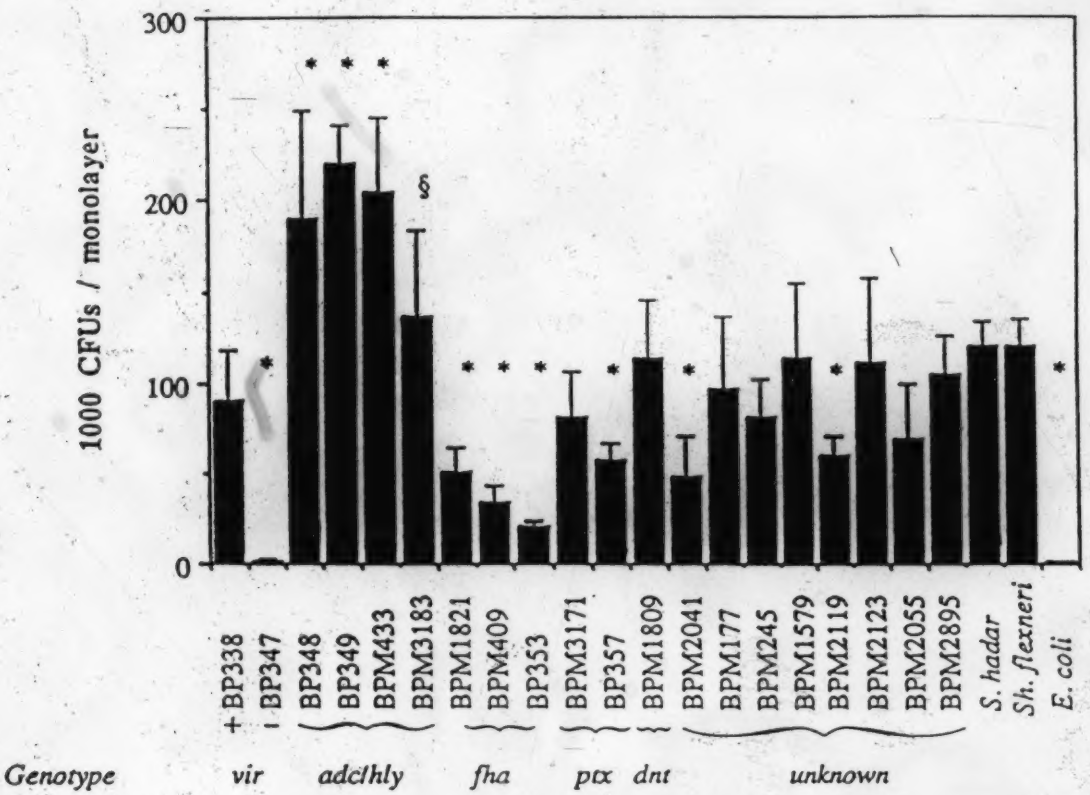

FIGURE III.2

Effect of exogenous FHA on invasion of parental BP338 (■), BP353 (FHA$^-$) (▨), and BP347 (vir-) (▨). *B. pertussis* were preincubated with FHA at the indicated concentrations, washed, then used in standard invasion assays. Each point represents an average of four independent determinations ± SD. *, statistically significant compared to untreated control at $P < 0.05$, §, statistically significant at $P < 0.025$.

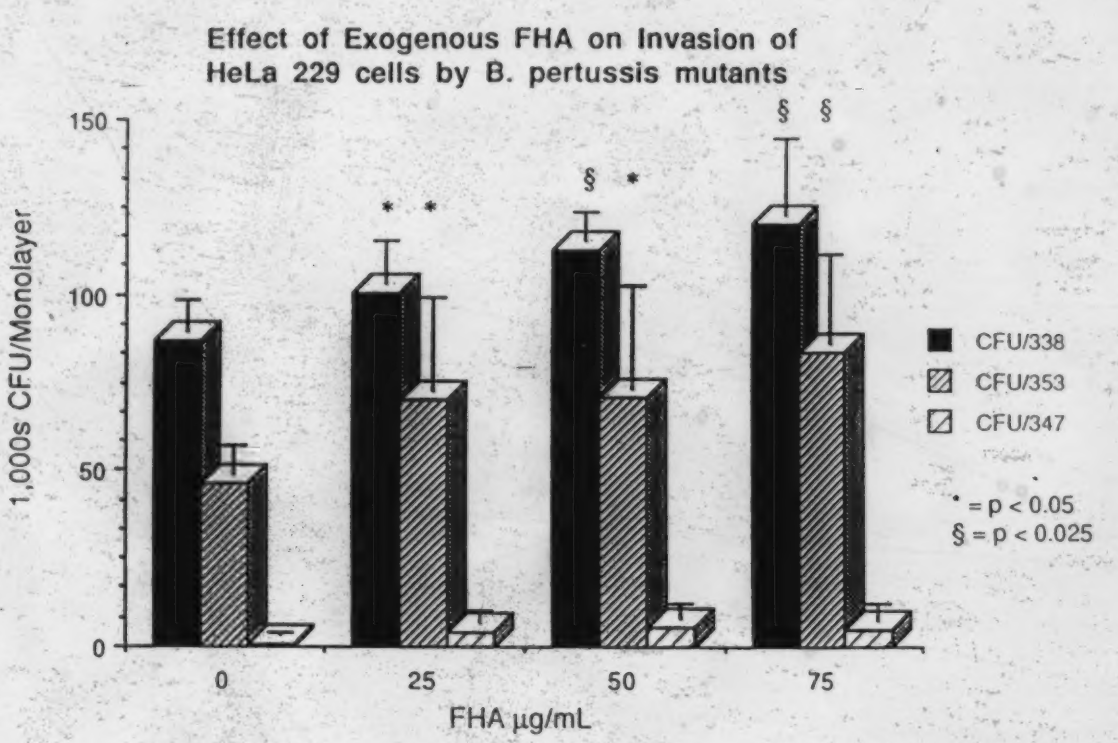
Effect of Exogenous FHA on Invasion of
HeLa 229 cells by B. pertussis mutants
1,000s CFU/Monolayer
150
100
50
0
0
25
50
75
FHA µg/mL
§ §
§ *
* *
CFU/338
CFU/353
CFU/347
* = p < 0.05
§ = p < 0.025

FIGURE III.3

Transmission electron micrographs demonstrating intracellular *Bordetella pertussis* within HeLa cells following a 7 hour coincubation period. (A)-(D) Parental BP338 within endocytic vacuoles. Note close proximity of bacterial outer membranes with membranes of vacuoles. (E) ACT/Hly$^-$ mutant BP348 during process of entry, whereupon outstretched microvilli of HeLa cell appear to engulf bacterium. (F) Intracellular BP348.

A
B
C

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Chapter IV

PHORBOL MYRISTATE ACTETATE-MEDIATED INHIBITION OF HELA 229 INVASION BY *BORDETELLA PERTUSSIS* AND OTHER INVASIVE PATHOGENS[1]

A. INTRODUCTION

The uptake of several known invasive bacterial pathogens by nonprofessional phagocytes is known to involve cytoskeletal actin microfilaments (1, 7, 15, 17). The invading bacterium initially adheres to the cell membrane, then transient actin polymerization at sites proximal to the site of entry is thought to trigger ingestion by the formation of a phagocytic vesicle enclosing the bacterium. F-actin is required for this process because bacterial uptake by a variety of tissue culture cell types is markedly reduced in the presence of cytochalasins, a group of fungal metabolites which bind to actin and modify its polymerization. For example, eucaryotic cell invasion by *Shigella*, *Salmonella*, *Yersinia* and *Bordetella* is cytochalasin-sensitive (4, 11, 12, 14). Both cytochalasins B and D have been used for this purpose; however, cytochalasin D is much more potent than cytochalasin B in terms of its effect on actin assembly.

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[1]A version of this chapter has been published. **Ewanowich, C. A. and M. S. Peppler**. 1990. Infect. Immun. 58:3187-3193.

This is likely due to the fact that cytochalasin D binds directly to the faster-growing (barbed) ends of actin filaments, whereas cytochalasin B binds to filament promoters distinct from those within the barbed ends (29). The disruption of actin architecture produced by cytochalasin treatment of cultured cells also disrupts their normal external morphology. For example, cytochalasins induce the formation of large numbers of bleb-like knobby protuberances at the cell membrane as a result of endoplasmic herniation (16), enucleation (6), and cellular rounding due to microfilament contracture and loss of substrate attachment sites (20). While such effects are of interest from a physiological point of view, they complicate interpretation of the effects of cytochalasins on bacterial invasion if they interfere with the ability of the bacteria to initally adhere to cultured cells.

The tumor promoter phorbol myristate acetate (PMA) causes numerous cellular changes through the activation of protein kinase C, among which is a profound and rapid alteration in cytoskeletal protein distribution. PMA treated cells rapidly lose their ordered actin stress fiber arrangement as filaments are displaced toward the periphery and appear in large ribbon-like networks at the cell margins. (10, 22, 24, 25, 26). Despite the alteration in actin distribution, however, the change in external appearance of cells treated with PMA for relatively short periods (ie. <12 hours) is limited to mild disruption of

cell-cell contacts and focal contacts, partial rounding, and acquisition of an irregular outline (19, 23, 24, 25), all of which are minor in comparison with the changes produced by cytochalasins B and D. Therefore, because PMA rapidly alters actin distribution without markedly altering external morphology so as to allow bacteria to properly adhere prior to invasion, it is a potentially useful reagent to investigate the microfilament-dependence of bacteria for invasion of eucaryotic cells.

In this chapter, we compare the effects of cytochalasins B and D, PMA, mezerein (a functional analog of PMA), and α4-phorbol-12,13 didecanoate (an inactive PMA analog) on actin distribution and bacterial adherence and invasion of HeLa 229 monolayers. PMA is shown to induce a significant change in HeLa cell actin distribution, but in contrast to cytochalasins B and D, it has no significant effect on gross cell morphology. The modified actin distribution is shown to reduce internalization of invasive bacterial pathogens such as *Bordetella pertussis*, *Yersinia pseudotuberculosis*, *Shigella flexneri* and *Salmonella hadar* in a dose-dependent manner at concentrations ranging from 1-1,000 ng/mL. Unlike cytochalasins B or D, PMA is shown to elicit a reduction in invasion without reducing adherence.

B. MATERIALS and METHODS

1. Bacterial Strains.

The nalidixic acid-resistant strain of *Bordetella pertussis* Tohama 1 (BP338) was kindly provided by Dr. Alison A. Weiss, Virginia Commonwealth University, Richmond, Virginia. The *Shigella*, *Salmonella* and *Yersinia* strains have been described previously in Chapters II and III. BP338 was cultivated on Bordet-Gengou agar with 13% sheep blood for 2 days prior to use in invasion assays. *Yersinia pseudotuberculosis* type A1, cultivated on brain heart infusion agar, was grown overnight in brain heart infusion broth at 28°C and recovered by centrifugation at 8,000 x g prior to use in invasion assays.

2. Fluorescence Labelling of F-actin and Vinculin.

To visualize F-actin, HeLa monolayers were grown in 4-well slides (Nunc, Naperville, ILL) and treated with drugs at the specified concentrations for 1 hour at 37°C. Monolayers were fixed with 3.7% formaldehyde for 10 minutes at room temperature, washed with phosphate buffered saline (54mM Na_2HPO_4, 13mM KH_2PO_4, 73mM NaCl), extracted with acetone at 4°C for 5 minutes, dried, then stained with 5 U/mL solution of F-actin-specific rhodamine-phalloidin (Molecular Probes, Inc., Junction City, OR.) in PBS for 30 minutes at room temperature. After washing, slides were mounted with a 90% glycerol/10% PBS solution and viewed

under a Leitz photomicroscope using epifluorescence with a 545nm excitation filter and 720nm emission filter.

For vinculin visualizaion a similar fixation/extraction procedure was followed. Following acetone extraction, cells were stained with mouse anti-chicken vinculin monoclonal antibody, clone VIN-11-5 (Sigma Chemical Co., St. Louis MO., (cross-reacts with human vinculin) for 30 minutes followed by FITC-conjugated goat anti-mouse (Sigma) for an additional 30 minutes. Slides were viewed using fluorescence filters for fluorescein (excitation at 490nm and emission at 525nm).

3. Treatment of HeLa cells and Invasion assay.

Phorbol myristate acetate (PMA), cytochalasins B and D, mezerein and α4-phorbol-12,13-didecanoate, all obtained from Sigma, were dissolved in either 100% ethanol (PMA) or dimethylsulfoxide (DMSO) (all others), and stored at -70°C. Reagents were added to HeLa monolayers in the indicated concentrations and incubated at 37°C for one hour prior to use in invasion assays. Control monolayers received DMSO or ethanol at concentrations equivalent to those found in monolayers incubated with the highest concentrations of drugs tested. Invasion assays were performed as previously described (12). The concentration of each of the drugs tested was maintained for the duration of each assay.

4. Adherence assays.

Adherence assays were performed as described for the invasion assay (12), except that monolayers were washed extensively and harvested for total counts following coincubation with the bacteria.

C. RESULTS

1. Effects of PMA and Cytochalasin B and D on Actin Distribution.

PMA and cytochalasins B and D promoted rapid changes in the distribution of F-actin in HeLa cells. Within untreated control HeLa cells (Fig. IV.1A), multiple stress fibers composed of F-actin were visualized spanning the cell. Vinculin associated with the ends of actin at focal contacts was distributed evenly throughout the cells (data not shown). Within one hour of treatment with PMA at 40ng/mL (Fig. IV.1B), stress fibers disappeared with the concurrent appearance of large actin bundles arranged as ribbons at the cell periphery. The normal polygonal outline of the cells was replaced by a more irregular one, marked by numerous lamellar protuberances. Vinculin was similarly concentrated at the cell periphery in conjuction with actin (data not shown). Pretreatment of HeLa cells with mezerein, a non-phorbol tumor promoter with protein kinase C activation ability similar to PMA (21), produced effects indistinguishable from PMA (Fig. IV.1D). In

contrast, the inactive PMA isomer α4-phorbol-12,13-didecanoate showed none of these effects (Fig. IV.1C). The effects of treatment with cytochalasins B and D were more dramatic. The most pronounced effects of cytochalasin B treatment of HeLa cells were the overall rounding of the cells and formation of spherical protrusions which stain avidly with phalloidin (Fig. IV.1E). Stress fibers were also present, but in reduced numbers compared to untreated controls. Cytochalasin D produced the most dramatic alterations in actin architecture as it transformed the ordered filar arrangement of actin into a disorganized, stellar-like punctate distribution (Fig. IV.F), which mirrored the marked cellular rounding and peripheral arborization seen under the inverted microscope (data not shown).

2. Effect of PMA on Bacterial Invasion of HeLa Monolayers.

To determine the effect of PMA on uptake of *Shigella flexneri*, *Salmonella hadar*, *Yersinia pseudotuberculosis* and *Bordetella pertussis*, semi-confluent monolayers were pretreated with PMA for one hour at concentrations ranging from 0.1-1,000ng/mL prior to addition of bacteria. The results are summarized in Figure IV.2, A-D. In each case, pretreatment of HeLa cells with PMA resulted in a dose-dependent reduction in invasion. At 1,000ng/mL PMA, the highest concentration tested, invasion of *Bordetella*

pertussis, *Salmonella hadar* and *Yersinia pseudotuberculosis* was reduced to a level of <15% of untreated controls. In contrast, the invasion of *Shigella flexneri* was more refractory to the inhibitory effect of PMA, as invasion was reduced to a level of 30% of control at a similar concentration of PMA. Figure IV.3 illustrates a similar dose-dependent reduction in uptake of BP338 in the presence of mezerein at concentrations ranging from 1-1,000nM, whereas PMA's inactive analog α4-phorbol-12,13 didecanoate was without effect on invasion at 1,000nM.

In separate experiments, neither PMA at 1,000ng/mL nor mezerein at 1,000nM reduced the viability of BP338 following an 8 hour coincubation period. Similarly, viability of the HeLa cells as assayed by trypan blue exclusion was not compromised following 8 hours coincubation with either PMA or mezerein at the concentrations indicated above.

3. Comparision of Effects of Cytochalasins B and D and PMA on Bacterial Invasion.

Table IV.1 presents a direct comparison of the ability of cytochalasins B and D and PMA to inhibit bacterial uptake by HeLa 229 monolayers. For each of the strains tested, cytochalasin D at 2.5μg/mL had the most pronounced inhibitory effect on invasion, reducing uptake to approximately 1% of untreated controls. In separate experiments using *S. flexneri* and *B. pertussis*, we

attempted to titrate cytochalasin D down to a concentration which elicited a significant reduction in invasion, yet preserved the cell morphology. We were not able to do so in either case, indicating that cytochalasin D's effects on actin are inseparable from morphological changes produced as a result of its actions. Cytochalasin B, which is significantly less potent than cytochalasin D in terms of its effects on actin, had varied effects depending on the species tested (Table IV.1). For example, cytochalasin B markedly reduced the uptake of *Bordetella pertussis* to a level comparable to that in the presence of an equivalent concentration of cytochalasin D (approximately 98% of control). In contrast, *Shigella flexneri* was significantly more refractory to the inhibitory effect of cytochalasin B at 2.5µg/mL than *B. pertussis*, *S. hadar* or *Y. pseudotuberculosis*, which also corresponded to the relatively lesser effect of PMA on *Shigella* uptake. The magnitude of the effect of PMA at 1,000ng/mL was intermediate between cytochalasin B and D, reducing uptake to approximately 15% of untreated controls for *B. pertussis*, *S. hadar* and *Y. pseudotuberculosis*, and 30% of untreated controls for *S. flexneri*.

4. Effect of PMA and Cytochalasins on Adherence of BP338 to HeLa monolayers.

Pretreatment of HeLa monolayers with either cytochalasin B or D results in pronounced alterations in

actin distribution and overall cellular morphology, especially in the case of cytochalasin D. We therefore found it necessary to determine whether these alterations reduced the numbers of bacteria which initally bound to the cells. Initally, adherence assays were performed at 4°C to abrogate uptake by the cell, so that the numbers of bacteria harvested after 5 hours would reflect extracellular adherent bacteria only, not a combination of extra- and intracellular bacteria. However, we found that *B. pertussis* does not form stable associations with the cell at 4°C, precluding quantitation of adherence in the absence of internalization at lower temperatures. Consequently, adherence assays were performed at 37°C, and the data presented in Figure IV.4 represents total associated CFU/monolayer (eg. intracellular + extracellular). For *B. pertussis*, the ratio of intracellular: extracellular CFU is low (eg. approximately $\leq$ 1:10), so that the values for untreated control, PMA, mezerein and α4-phorbol-12,13-didecanoate, which reflect numbers of cell-associated organisms, do not change significantly. Thus, Figure IV.4 illustrates that adherence of *B. pertussis* is unaltered in the presence of the highest concentrations of PMA, mezerein or α4-phorbol-12,13-didecanoate tested. In contrast, adherence in the presence of cytochalasins B and D at 2.5μg/mL is reduced to approximately 50% of control. This finding was expected based on the dramatic morphological alterations of HeLa

cells elicited by both cytochalasin B and D mentioned above. Similar data were obtained enumerating fluorescent bacteria adherent to HeLa monolayers directly under a fluorescence microscrope (data not shown).

D. DISCUSSION

A characteristic common to several invasive bacterial pathogens is the ability to induce a transient rearrangement of the F-actin architecture at sites proximal to entry in nonprofessional phagocytic cell types (7, 15). The stimulus that induces this rearrangement is unknown, although it may involve differential phosphorylation of a ubiquitous class of transmembrane receptors concentrated in focal contacts known as integrins. These receptors appear to transduce signals directly from the cell membrane to the actin network via intermediate proteins such as talin, vinculin and α-actinin, as depicted in Figure IV.5 (5, 8, 9, 13, 18, 27, 28).

The requirement for F-actin in bacterial uptake by nonprofessional phagocytes has been demonstrated using several different techniques. In 1987, Clerc and Sansonetti (7) demonstrated this requirement using two novel methods. One involved the use of phallacidin, a phallotoxin which specifically binds F-actin, linked to a fluorescent dye. Using this reagent, Clerc and Sansonetti observed fluorescent aggregates of filamentous actin

beneath the membrane of HeLa cells whose appearance coincided with the penetration of invasive *Shigella flexneri*. Such a technique was later used by Finlay and Falkow (15) to demonstrate the transient appearance of F-actin upon penetration of Madin-Darby Canine Kidney cells by invasive *Salmonella*. In addition, Clerc and Sansonetti directly quantitated a decrease in the monomeric (G): total (F+G) actin ratio upon penetration of *Shigella flexneri*, which reflected *de novo* polymerization of monomeric actin induced by the invading bacteria (2, 3, 7).

To date, however, the majority of studies that demonstrate a requirement for filamentous actin in bacterial uptake have utilized cytochalasins B and D. Although these drugs bind specifically to F-actin, their actions result in corresponding changes in cell morphology which interfere with the ability of invading bacteria to first adhere to the plasma membrane, as shown in this study. Consequently, the reduced numbers of intracellular CFU's obtained following cytochalasin treatment do not accurately reflect the reduction due to microfilament disruption alone.

The tumor promoter PMA causes a rapid and dramatic alteration in actin distribution in numerous cell lines (10, 22, 25, 24, 25, 26), including HeLa cells, as demonstrated in this study. Unlike cytochalasins, however, treatment of HeLa cells up to a PMA concentration of 1,000ng/mL resulted in only minor changes in cell

morphology compared to the cytochalasins. Therefore, we decided to determine whether PMA would reduce invasion of several different bacterial pathogens, as a possible alternative to the use of cytochalasin B or D for delineating the microfilament requirement for uptake.

The data presented here demonstrate a dose-dependent reduction in uptake of virulent *B. pertussis*, *S. flexneri*, *S. hadar*, and *Y. pseudotuberculosis* by HeLa cells following one hour incubation with PMA at concentrations ranging from 0.1 to 1,000ng/mL. A similar reduction in uptake of *B. pertussis* was demonstrated with a functional analog of PMA, mezerein (21), whereas an inactive isomer, α4-phorbol-12,13-didecanoate, was without effect on invasion. Cytochalasin D was the most potent drug in terms of its effects on actin and reductive effect on invasion; however, cytochalasin D also causes the most disruption of cellular morphology. The reduction in invasion elicited by an equivalent concentration of cytochalasin B varied according to the species tested. The invasion of *B. pertussis*, at one extreme, was reduced to approximately 99% of untreated control, whereas *S. flexneri* was more resistant to its effects; reduced to only 50% of control. PMA at 1,000ng/mL elicited a more uniform reduction in uptake among the species tested, which was intermediate in magnitude between cytochalasins B and D. Similar to the result obtained with cytochalasin B, *S. flexneri* was also more refractory to the reductive effect of PMA. Perhaps the

differences in susceptibility to cytochalasin B and PMA demonstrated by the species tested reflect differences in relative requirements for actin in uptake by nonprofessional phagocytes.

An important finding was that the profound alterations in cellular morphology and the actin network elicited by both cytochalasins B and D caused not only a reduction in invasion, but also a significant reduction in adherence. In contrast, we demonstrate that PMA caused a significant reduction in invasion without affecting adherence. This is important because it permits study of the invasion process without affecting adherence. One potential disadvantage to the use of PMA is its lack of specificity, as it may interfere with other cellular processes required for subsequent intracellular survival, receptor synthesis/ recycling or overall cellular homeostasis if present for an extended period of time.

To summarize, this study introduces a novel reagent for use in examining the bacterial requirement for microfilaments in uptake by cultured cells. PMA is well-known to cell biologists for its effects on the actin cytoarchitecture of cultured cells which resemble those produced by tumor viruses. It is similarly capable of inhibiting the uptake of several different species of invasive bacteria through its effects on actin distribution. Importantly, it does so without causing deterioration of cellular morphology as do the

traditionally used cytochalasins B and D, thereby permitting bacterial adherence, the first of the sequence of events leading to uptake, to proceed unhindered.

TABLE IV.1.

Comparison of Efficacy of Agents Used to Inhibit Bacterial Invasion of HeLa 229 Monolayers[a]

	Organisms/Monolayer (% of Control)		
	Cy B 2.5 µg/mL	CyD 2.5 µg/mL	PMA[b] 1.0 µg/mL
B. pertussis	1.7 ± 2.4	1.4 ± 0.5	11.8 ± 2.1
S. flexneri	50.9 ± 16.3	1.1 ± 1.8	28.9 ± 6.7
S. hadar	26.8 ± 21.6	0.3 ± 0.2	14.7 ± 6.3
Y. pseudo-tuberculosis	28.3 ± 17.7	0.1 ± 0.1	11.6 ± 6.8

a. Results represent the average number of CFUs per monolayer expressed as a percentage of control ± standard deviation; n=3 independent determinations for each.
b. Monolayers were preincubated with cytochalasins B or D or PMA at the concentrations indicated above for 1 hour prior to addition of bacteria. Numbers of intracellular bacteria were quantitated using the gentamicin assay as described previously (Ewanowich *et al.*, 1989).

FIGURE IV.1

Rhodamine-phalloidin flourescence labelling of F-actin in HeLa 229 monolayers (400x magnification). Monolayers were incubated with no additions (control, A) or PMA at 40ng/mL (B), α4-phorbol-12,13-didecanoate at 1,000nM (C), mezerein at 1,000nM (D), cytochalasin B at 2.5µg/mL (E) or cytochalasin D at 2.5Mg/mL (F) for one hour at 37°C prior to processing as described in Materials and Methods.

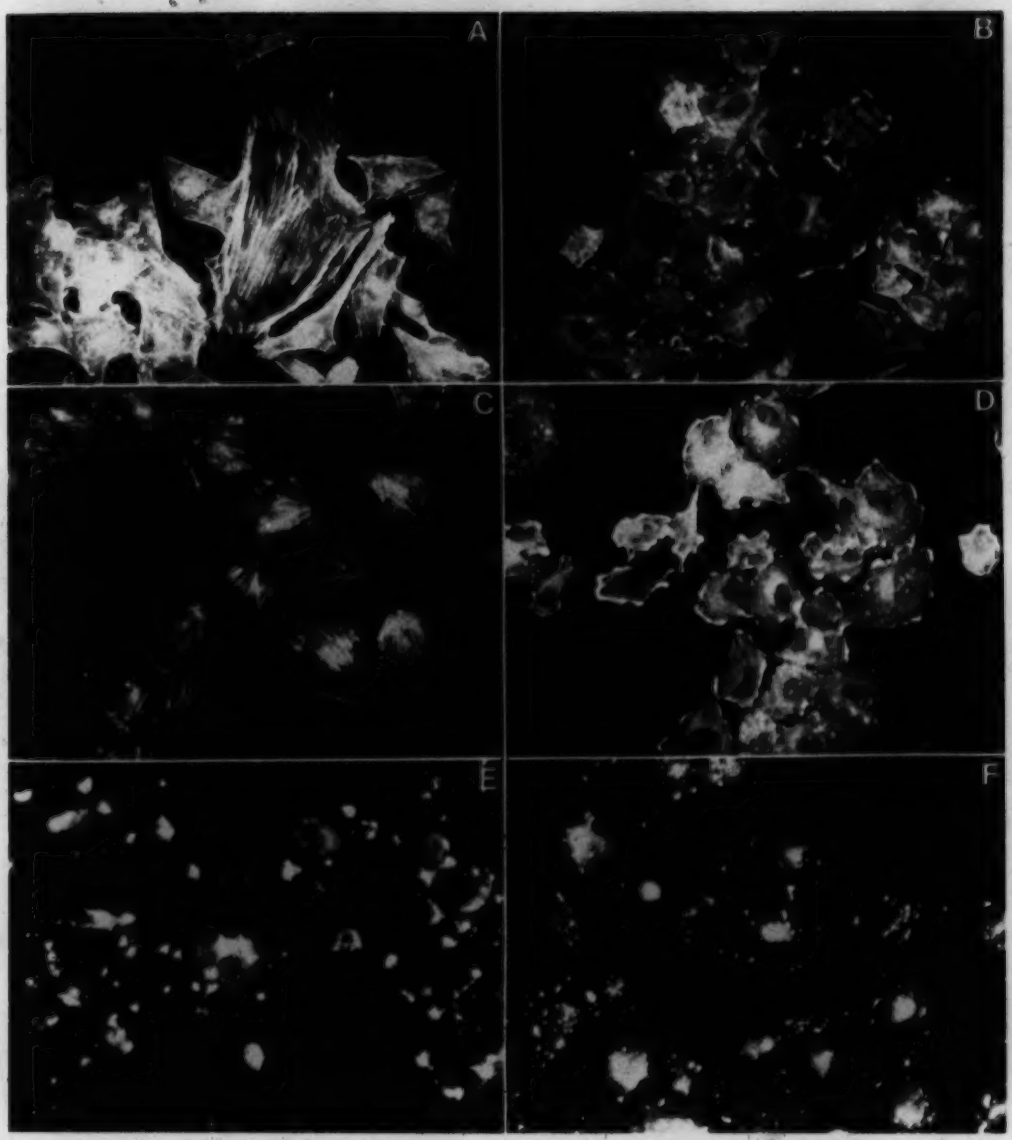

A
B
C
D
E
F

FIGURE IV.2

Dose-dependent inhibition of invasion of HeLa 229 monolayers by various invasive bacteria mediated by PMA. (A) *B. pertussis* strain 338, (B) *Shigella flexneri* serotype 0:3 E2549, (C) *Salmonella hadar*, (D) *Yersinia pseudotuberculosis* Type A1. Values represent the mean ± standard deviation CFU (in thousands) recovered from gentamicin-treated monolayers from 3 independent determinations. HeLa monolayers were incubated with PMA at the indicated concentrations for 1 hour prior to addition of bacteria.

Dose-Dependent Inhibition of Invasion of HeLa 229 Monolayers by Various Invasive Bacteria Mediated by PMA

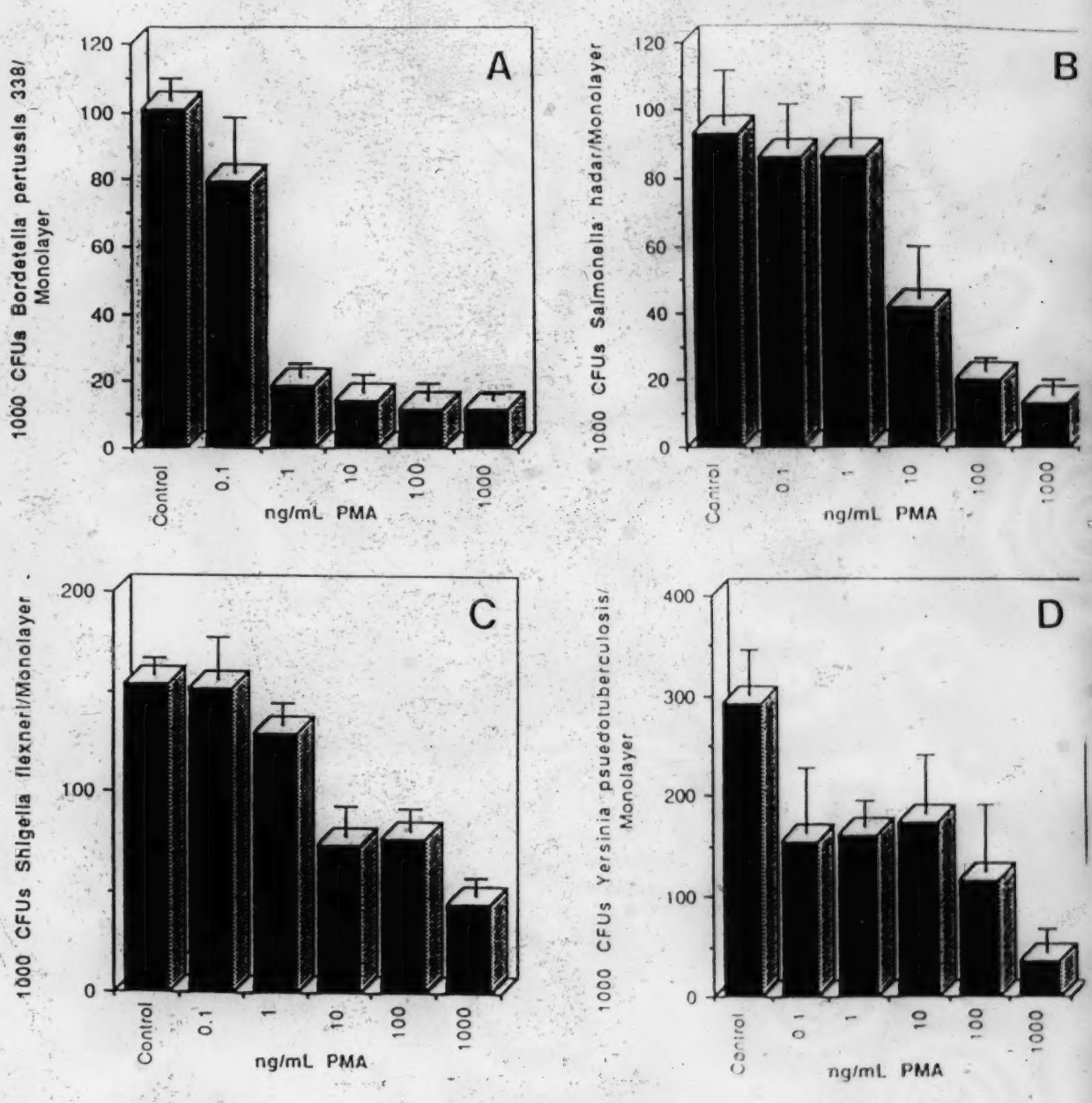


FIGURE IV.3

Effect of mezerein and α4-phorbol-12,13-didecanoate (α4PDD) on uptake of *B. pertussis* 338 by HeLa 229 monolayers. Each datum point represents mean ± standard deviation of gentamicin-resistant CFU (in thousands) from 3 independent determinations.

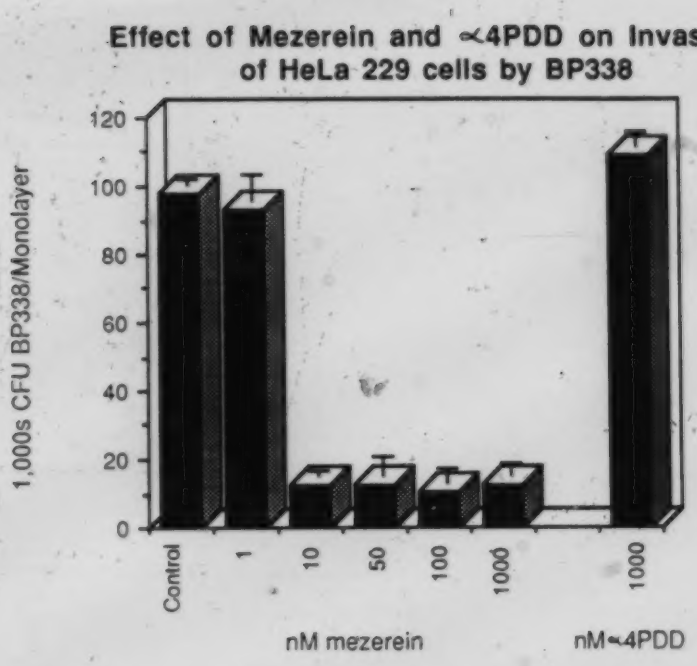

Effect of Mezerein and ∝4PDD on Invasion of HeLa 229 cells by BP338
1,000s CFU BP338/Monolayer
120
100
80
60
40
20
0
Control
1
10
50
100
1000
1000
nM mezerein
nM∝4PDD

FIGURE IV.4

Effect of PMA, mezerein, cytochalasins B and D, and α4PDD on adherence of *Bordetella pertussis* 338 at highest concentrations tested in assays. Data are expressed as % of untreated controls and represent mean ± standard deviations from 3 independent assays. $; statistically signficant at $P < 0.0005$ compared to untreated controls.

Effect of Microfilament Disruptors on Adherence of BP338 to HeLa 229 cells

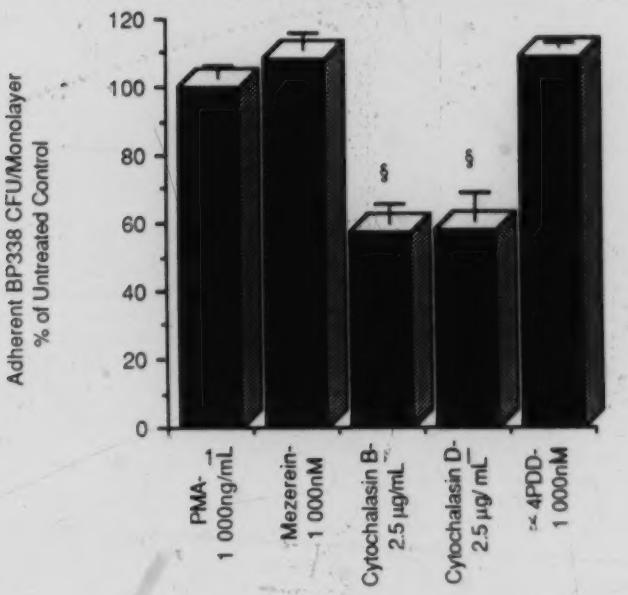
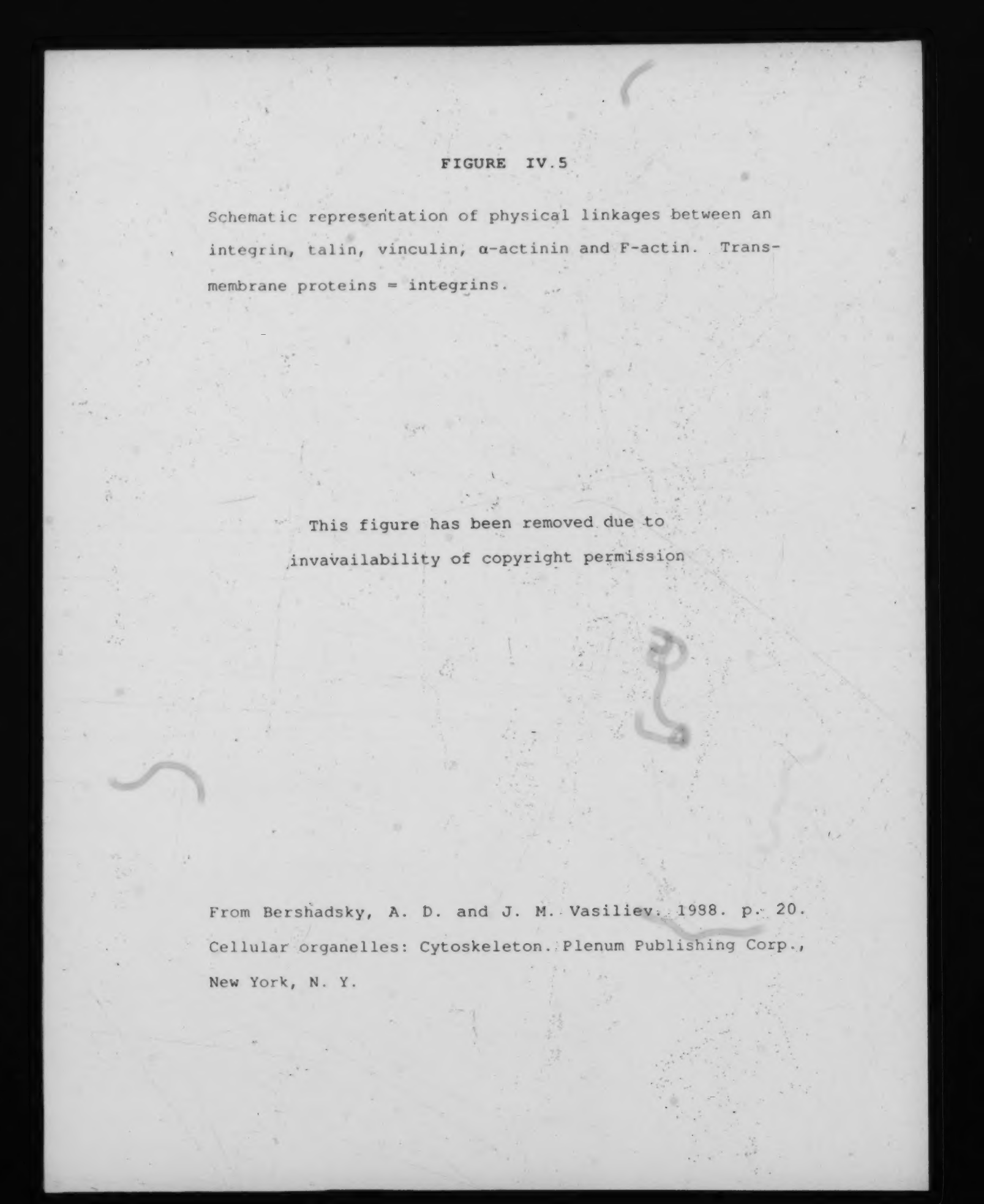


FIGURE IV.5

Schematic representation of physical linkages between an integrin, talin, vinculin, α-actinin and F-actin. Transmembrane proteins = integrins.



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Chapter V

ROLE OF A 69 KDA OUTER MEMBRANE PROTEIN IN THE INVASION OF HELA CELLS BY *BORDETELLA PERTUSSIS*[1]

A. INTRODUCTION

This chapter examines the role of pertactin (19) on *Bordetella* invasiveness. Pertactin is a virulence-associated, surface-exposed 69 kDa protein found on *B. pertussis* which can elicit a protective immune response in animal models (4, 30). Although there exists some evidence that links pertactin to the adenylate cyclase toxin of *B. pertussis* (22, 23), the true function of pertactin is as yet unknown.

The entire nucleotide sequence of pertactin has recently been published (10), and its derived amino acid sequence contains two Asp-Gly-Arg (RGD) sequences. This is significant because the presence of an RGD sequence(s) within an adhesive ligand is often associated with the ability of the ligand to recognize a class of receptors on mammalian cells known as integrins (26). Integrins are large, αβ heterodimeric integral membrane glycoproteins which interact with a wide array of extracellular matrix

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[1] One version of this chapter is in press and another has been submitted: **Ewanowich, C. A. and M. S. Peppler.** 1990. Proc. Sixth Int. Symp. Pertussis, Bethesda, Maryland, and **Leininger, E., C. A. Ewanowich, M. S. Peppler, J. G. Kenimer and M. J. Brennan,** respectively.

proteins, including fibronectin, collagen, fibrinogen, vitronectin, von Willebrand factor, laminin and thrombospondin (14, 29). Since each β chain can associate with several different α chains, integrins are classified according to the identity of their β chain into several subfamilies (eg. β1, β2, β3 etc.) each member of which shares a common β chain (14, 29). Representative subfamilies include the VLA or β1 subfamily, including the fibronectin receptor; the leu-CAM subfamily, including MAC-1 (C3Bi), p150,95 and LFA-1; and the cytoadhesin family, including platelet-derived glycoprotein IIb-IIIa and the vitronectin receptor (Table V.1).

In addition to extracellular adhesive proteins, integrins also appear to recognize sequences within bacterial outer membrane proteins which promote both adherence and internalization. Earlier this year, Isberg and Leong demonstrated that invasin, an outer membrane protein of *Yersinia pseudotuberculosis* which promotes entry of the bacteria into mammalian cells (16), specifically bound to at least four different integrins belonging to the VLA subfamily, including the fibronectin receptor (15). Unlike most integrin-specific ligands, invasin does not contain an RGD sequence (17), although in keeping with their observation that Mabs directed against the fibronectin receptor block attachment of both fibronectin and invasin, Isberg and Leong suggested that invasin may contain the same structural information as is found in RGD-

containing ligands (17). Later the same year, Relman *et al.* (28) demonstrated that *B. pertussis* adheres to the β2 integrin known as CR3 or MAC-1 on human macrophages. CR3 recognizes an RGD sequence in the complement ligand C3bi (32). Relman *et al.* showed that attachment of *B. pertussis* to CR3 is mediated at least in part by an RGD sequence within FHA. They pointed out that many intracellular parasites of macrophages utilize integrins for entry, the predominant integrin being CR3 (7, 20, 25). They also suggested that CR3 is the integrin of choice for ligation by intracellular parasites because, while interaction with CR3 induces internalization, it does not activate mechanisms which initiate intracellular killing (34). This can provide the parasite with a convenient means of entering macrophages without the suicidal consequences, while further allowing the organism a protected intracellular reservior.

Detection of two RGD sequences within pertactin led us to examine the potential role of pertactin in invasion of HeLa cells by *B. pertussis*, as outlined in this chapter.

B. MATERIALS AND METHODS

1. Bacteria and Growth Conditions

BP338, a nalidixic acid-resistant strain of *B. pertussis* Tohama 1, was obtained from Dr. Alison A. Weiss, Department of Microbiology and Immunology, Virginia

Commonweath University, Richmond, Viriginia. *B. pertussis* strain 18323 derivatives 868 (parent), 870 (Pertactin, Pn^-), 872 (FHA^-) and 875 (Pn^-, FHA^-) (Table V.2) were obtained from Dr. Michael Brennan, Division of Bacterial Products, Center for Biologics Evaluation and Research, Food and Drug Adminstration, Bethesda, MD. Because strains 872 and 875 contained a gentamicin resistance cassette in one of the structural genes for FHA (*fhaB*), the invasive capacity for this series of mutants was assessed using polymyxin B at 100µg/mL to select against viable extracellular bacteria. After 2 hours incubation at 37°C at a concentration of 100µg/mL, polymyxin B was shown to kill aproximately 99.9999% of a population of *B. pertussis* containing an equivalent number of organisms inoculated into HeLa monolayers in the assay described below. Bacteria were routinely cultivated on Bordet-Gengou agar (BGA) supplemented with 15% sheep blood at 37°C in a humidified atmosphere. For use in invasion assays, bacteria grown two days on BGA were suspended in Hanks balanced salt solution containing 25mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.2) (Hanks-HEPES) to an optical density of 0.12 at 540nm and diluted appropriately.

2. Invasion assays.

HeLa 229 cells (human epithelium-like; ATCC CCL 2.1) were used in invasion assays as previously described in Chapter III. In assays using gentamicin-resistant mutants,

polymyxin B at a concentration of 100µg/mL was used in place of gentamicin. After 2 hours, the monolayers were washed and harvested for quantitation of viable intracellular CFUs.

3. Inhibition of Invasion by Synthetic Peptides.

Synthetic peptides containing RGD regions of pertactin, fibronectin and filamentous hemagglutinin were kindly donated by Drs. Elizabeth Leininger and Michael Brennan, Division of Bacterial Products, Center for Biologics Evaluation and Research, Food and Drug Adminstration, Bethesda, MD. The sequences of these peptides are listed in Table V.3.

Immediately prior to use in invasion assays, peptides were diluted in MEM/3% fetal bovine serum to obtain final concentrations of 1 and 10µg/mL. Monolayers were incubated with 0.4mL of diluted peptide for 1 hour at 37°C. Following preincubation with the appropriate peptide, monolayers were used in the invasion assay as described above. Due to their reversibility of binding, peptides were present during the entire assay at the concentrations indicated above.

4. Effect of Monoclonal Antibody F6E5 on Invasion.

The antibody F6E5 specific for P1 was also obtained from Dr. Michael Brennan, Food and Drug Administration. It was diluted in suspensions of BP338 in Hanks-HEPES to

obtain final concentrations of 0.05 and 0.1mg/mL and incubated at 37°C for 1 hour to allow binding. After 1 hour, bacteria were washed once, diluted appropriately, then used in invasion assays as described above.

C. RESULTS

1. Effect of synthetic peptides on *B. pertussis* invasion of HeLa cells.

In a recent report (19), Leininger *et al.* demonstrated that purified pertactin coated onto plastic tissue culture wells promoted the attachment and spreading of Chinese Hamster Ovary (CHO) cells. This interaction could be specifically inhibited by a synthetic peptide containing a region homologous to that found on pertactin containing an RGD sequence. In addition, a mutant which did not express pertactin was significantly less able to adhere to CHO and HeLa cells than the parent strain. Consequently, because pertactin plays a putative role in the adherence of *B. pertussis* to mammalian cells and contains functional RGD sequences reminiscent of those found on other extracellular matrix proteins, we wanted to examine the possiblity that pertactin might similarly mediate invasion by *B. pertussis*.

We first evaluated a number of extant peptides for their ability to inhibit invasion of HeLa cells by *B. pertussis* by competing for specific receptors on the surface of the eukaryotic cells with the bacteria. It was

necessary to limit the concentrations of peptides incubated with the HeLa cells, as higher concentrations (eg. 500 µg/mL) of some peptides containing RGD sequences (especially those derived from fibronectin) caused HeLa cell perimeter to lift off the well-bottom, producing a rounded appearance. This effect was fully reversible if the peptide was removed from the incubation medium. This was presumably caused by competition for receptors on the HeLa membrane between the peptide and unnamed factors used by the manufacturers to pretreat the well surface, favoring the soluble peptide at higher concentrations. Peptide concentrations of 1 or 10 µg/mL used herein did not cause notable rounding of the HeLa cells. Assuming a molecular weight of approximately 1200 daltons, at a concentration of 1 µg/ml, the calculated mole ratio of soluble 14-mer peptides to HeLa cells is approximately $4 \times 10^{15} : 1$.

The results described in Figure V.1 indicate that the only peptide able to elicit a significant reduction in invasion is P1, the first sequential RGD tripeptide found in the pertactin sequence. A significant reduction (45.2% compared to the untreated control) was found with a peptide concentration as low as 1 µg/mL. A single amino acid replacement of the aspartic acid residue of RGD with a glutamic acid charge analog in the RGE tripeptide in P3 completely abrogated the ability of the peptide to complete for adherence to the HeLa receptor(s). In contrast, the second RGD sequence in pertactin represented by peptide P2

had no signficant effect on invasion. Likewise, peptides containing representative RGD sequences found in FHA and fibronectin did not signifcantly affect internalization.

2. Effect of a monoclonal antibody to the P1 sequence in pertactin on the invasion of *B. pertussis*.

In addition to the pertactin-specific monoclonal antibodies described in Chapter III, we examined an additional antiserum which has been reported to inhibit adherence of CHO cells to purified preparations of pertactin (Leininger, unpublished observations). While the exact specificities of these antisera are unknown, the precise location of the epitope within pertactin recognized by F6E5 has been localized to the RGD sequence of P1 (Michael Brennan, unpublished data). This antibody was able to significantly reduce HeLa invasion by *B. pertussis*, as indicated in Figure V.2. Invasion was reduced by 42.2% and 35.5% compared to untreated controls in the presence of 0.05mg/mL and 0.1mg/mL antibody, respectively, lending further support for a role for P1 in HeLa invasion.

3. Comparison of the abilities of mutants lacking pertactin and/or FHA to invade HeLa monolayers.

We further evaluated the differential roles of pertactin and FHA in invasion using a series of mutants of *B. pertussis* strain 18323. Since two of these mutants were

rendered gentamicin-resistant by the presence of a gentamicin resistance cassette inserted into a structural gene for FHA, gentamicin could not be used to destroy extracelluar organisms in assays using these mutants. Instead, polymyxin B at a concentration of 100 µg/mL was used in the same fashion as gentamicin. Previous experience with polymyxin B indicated that the number of viable BP338 organisms recovered following incubation with this antibiotic was normally up to 50% higher than that recovered after gentamicin treatment (data not shown). Since both antibiotics destroy approximately equal numbers of viable *B. pertussis* over a two-hour interval, the lower number of organisms recovered following gentamicin treatment may reflect some degree of killing of intracellular bacteria by residual gentamicin which gains entry via pinocytosis and still maintains some degree of activity. In addition, due to undefined differences among strains of *B. pertussis*, the numbers of viable 18323 organisms recovered following gentamicin treatment are consistently 20-40% lower than BP338 (data not shown). The difference in magnitude of invasion of parent strain 868 shown in Figure V.3 compared to the magnitude of BP338 invasion previously reported (Chapter IV) can be thus attributed to both degree of invasiveness (strain-dependent) and polymyxin B activity.

As shown in Figure V.3, no significant difference (*t*-test) existed between invasion of the parent strain 868 and

870, the pertactin mutant. As expected based on previous data, invasion of the FHA mutant 872 was reduced compared to the parental control ($P < 0.01$, student's t-test). Mutant 875 lacking both Pn and FHA was also reduced compared to the control ($P < 0.1$), which was most likely due solely to a lack of FHA in view of the behavior of strains 870 and 872. Interestingly, statistical anaylsis revealed also a significant difference between invasion of strains 872 and 875 ($P < 0.05$).

D. DISCUSSION

Dr. Stanley Falkow once drew an apt and illustrative analogy between the process of bacterial invasion of nonphagocytic mammalian cells and the operation of an elevator (Symposium presentation, Annual Meeting of the American Society for Microbiology, Anaheim, CA, 1990). In it, the invasive pathogen was able to "push the correct button" which summoned the "elevator" to the cell surface, thereby gaining access to the cell's interior. Unlike invasive pathogens, noninvasive pathogens and commensals were unable to "press the button", and were thus denied the opportunity for entry. Of course, gaining entry into the cell was only the first of several steps an intracellular pathogen would be required to execute in order to be successful, as intracellular killing would eventually

follow unless the organism possessed a means by which it could thwart the destructive mechanisms of the host cell.

The identification and characterization of this "button" has been the subject of intense research effort during the past few years. The major candidates for such a role are members of the integrin superfamily, so named to denote their involvement as integral membrane links between the extracellular matrix and intracellular actin cytoskeleton (31). Integrins and other molecules such as actin, talin (8), vinculin (9) are concentrated in areas of the cell membrane which adhere to the extracellular matrix known as focal contacts or adhesion plaques (6, 11). Adhesion plaques are believed to be the site of signal transduction from the extracellular matrix to intracellular cytoskeleton via resident transmembrane integrin molecules. The extracellular domain of the integrin receptor receives a message from an external ligand, which appears to be transferred to the cytoskeleton through sequential associations between talin (33), then vinculin (24), and finally α-actinin (2), which is believed to associate with actin filaments, nucleating their assembly (see Figure IV.5, Chapter IV). Although the precise mechanisms of signal transduction involving integrins and associated proteins are not completely understood, they are believed to involve differential phosphorylation of a conserved tyrosine residue on the cytoplasmic domain of the integrin β chain by activated protein kinases once its extracellular

domain is occupied by a suitable ligand (31). One functional consequence of integrin occupation on polymorphonuclear leukocytes and monocytes is phagocytic engulfment (5, 27, 33). Here lies the root of interest in integrins from the point of view of an invasive bacterial pathogen, for such an organism could conveniently and easily signal its desire to be internalized to the cytoskeleton of a nonphagocytic cell simply by occupying the correct site within a functional integrin receptor.

In light of this form of transmembrane communication mediated by integrins, the need to explore the possible involvement of pertactin in invasion of nonprofessional phagocytes by *B. pertussis* became evident when it was recently reported that pertactin contains two RGD tripeptides within its sequence 10). P2, the second RGD sequence in pertactin, appears to be removed post-translationally (Ian Charles, personal communication). The data described here demonstrate that the presence of a soluble 14-mer peptide containing an RGD sequence corresponding to the first predicted RGD sequence in pertactin inhibits invasion of HeLa cells by *B. pertussis*. This presumably results from competition between the soluble peptide and a corresponding sequence on pertactin for identical receptors on the HeLa surface. Although the involvement of an RGD sequence hints that the unidentified receptor may be an integrin, there are no data as yet to support this claim. The specificity of inhibition was

illustrated by the fact that it was abrogated by the conservative substitution of a single amino acid substitution (Asp→Glu) within the 14-mer. Futhermore, peptides corresponding to RGD regions in both fibronectin and filamentous hemagglutinin had no effect on *B. pertussis* invasion, indicating that pertactin occupies a HeLa receptor distinct from those which recognize either of these molecules.

To futher support the involvement of the RGD sequence contained within P1 of pertactin in the invasion of HeLa cells by *B. pertussis*, we demonstrate that organisms incubated with a monoclonal antibody specific for the P1 region of pertactin are significantly impaired in their ability to invade HeLa cells.

The fact that the presence of exogenously supplied pertactin sequences elicited a significant inhibition of invasion is difficult to reconcile with the observation that a mutant unable to express functional pertactin was not significantly impaired in its ability to invade. In view of data presented in a recent report by Lee *et al.* (18), a potenial source for this descrepancy may be related to the use of polymyxin B to select against extracellular bacteria. Using polymyxin B to destroy extracellular bacteria in a similar assay, Lee *et al.* reported results which directly conflicted with those we reported obtained from invasion assays using gentamicin to select against extracellular bacteria (Chapters II and III). Since these

assays differed only in the selective antibiotic employed and the methods used to disperse HeLa monolayers, it seems likely that the use of a membrane-reactive antibiotic such as polymyxin B is at least partly responsible for the differences in data obtained. Perhaps this series of mutants should be reevaluated for differences in invasion using a less reactive antibiotic to destroy extracellular organisms.

Another possible explanation is that the antibiotic resistance determinant inserted into the pertactin sequence at an unmapped location disturbed the epitope used to screen the mutants for absence of immunological activity (a monoclonal preparation which recognized an unknown epitope within pertactin was used to select mutants), while preserving the RGD sequence required for biological activity. In such a case, it would be necessary to isolate the protein product of the interrupted pertactin gene and examine it for biological activity, viz, ability to promote adherence and spreading of mammalian cells.

One possible source of confusion regarding the data obtained using this series of FHA/Pn mutants lies in their parental strain 18323. Unlike other strains of *B. pertussis* tested to date, strain 18323 is remarkably virulent for mice when injected intracerebrally; which forms the basis for its use in the intracerebral challenge test for vaccine efficacy (1, 3). Furthermore, multilocus enzyme electrophoresis used to assess genetic diversity in

Bordetella sp. indicated that *B. pertussis* strain 18323 is genetically more closely related to *B. bronchiseptica* than other strains of *B. pertussis* (21). For these reasons, it would be more applicable to study the roles of FHA and Pn in *B. pertussis* adherence and invasion in a background which better typifies the "average" strain of *B. pertussis*.

It is apparent that invasion by *B. pertussis* is both mutifactorial and involves some degree of redundancy. That pertactin assumes an integral role in this process is a viable possibility, as indicated by the data described here, and therefore demands further investigation. The logical first step in such an investigation is the identification and characterization of the corresponding receptor on HeLa cells which binds pertactin to determine whether it is a member of the integrin superfamily. A similar approach to that used by Relman *et al.* to demonstrate that adherence of *B. pertussis* to human macrophages occurs via a specific interaction between macrophage integrin CR3 and an RGD-containing region of FHA (28) could be employed. To begin, a decrease in invasion following incubation of HeLa cells with monoclonal antibodies specific for the β chain of the integrin cytoadhesin family would suggest a role for an integrin receptor. The specific type of receptor within the cytoadhesin class could be determined by determining the effect of monoclonal antisera against integrin-specific α chains. Finally, HeLa cell variants lacking specific

integrin receptors could be utilized to confirm the speoificity of binding.

TABLE V.I

The Integrin Family of Adhesion Molecules

This figure has been removed due to invavailability of copyright permission.

From: Elisabetta, D., and D. Lauri. 1990. Biochemical and functional characteristics of integrins: A new family of adhesive receptors present in hematopoietic cells. *Haematologica* 75:1-6.

TABLE V.2

List of *B. pertussis* 18323 Mutant Strains Used in this Study

Strain	Relevant property	Source
BP 868	Parent	M. J. Brennan
BP 870	PN^{-a}	M. J. Brennan
BP 872	FHA^{-b}	M. J. Brennan
BP 875	PN^{-}, FHA^{-}	M. J. Brennan

[a] PN, pertactin
[b] FHA, filamentous hemagglutinin

TABLE V.3

Synthetic Peptides Used for Inhibition of HeLa cell Invasion by *Bordetella pertussis*

Name (abbreviation)	Sequence
Pertactin 1 (P1-RGD)	ATIR**RGD**ALAGGAC
Pertactin 2 (P2-RGD)	AGYT**RGD**RGFTGDC
Pertactin 3 (P3-RGE)	ATIR**RGE**ALAGGAC
Filamentous hemagglutinin 1 (FHA-RGD)	VTVG**RGD**PHQGVLC
Fibronectin RGD (FN-RGD 6aa)	G**RGD**SP
Fibronectin RGD (FN-RGD 14aa)	AVTG**RGD**SPASSKC
Fibronectin RGE (FN-RGE 6aa)	G**RGE**SP

FIGURE V.1

The effect of synthetic peptides containing RGD sequences of pertactin (PRN), FHA and fibronectin (FN) on the ability of *Bordetella pertussis* strain BP338 to invade HeLa cells. The identity and sequences of the peptides are listed in Table V.3. The values plotted represent the mean ± standard deviation for gentamicin-resistant CFUs from three independent determinations. $ = $P \leq 0.0005$ compared to untreated controls (t-test).

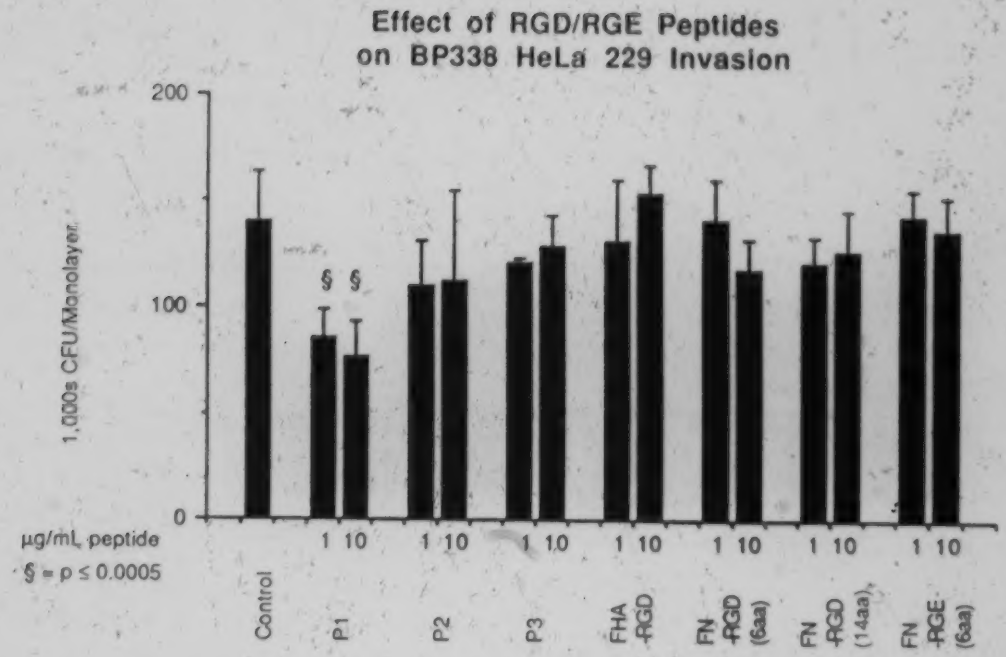

Effect of RGD/RGE Peptides
on BP338 HeLa 229 Invasion
200
100
0
1,000s CFU/Monolayer
§ §
µg/mL peptide
§ = p ≤ 0.0005
1 10
Control
P1
P2
P3
FHA RGD
FN RGD (6aa)
FN RGD (14aa)
FN RGE (6aa)

FIGURE V.2

The ability of pertactin-specific monoclonal antibody F6E5 to inhibit invasion of HeLa cells by *B. pertussis* strain 338. The plotted values represent the mean ± standard deviation of gentamicin-resistant CFUs recovered from three independent determinations. § = $P \leq 0.005$, * = $P \leq 0.0005$ (*t*-test).

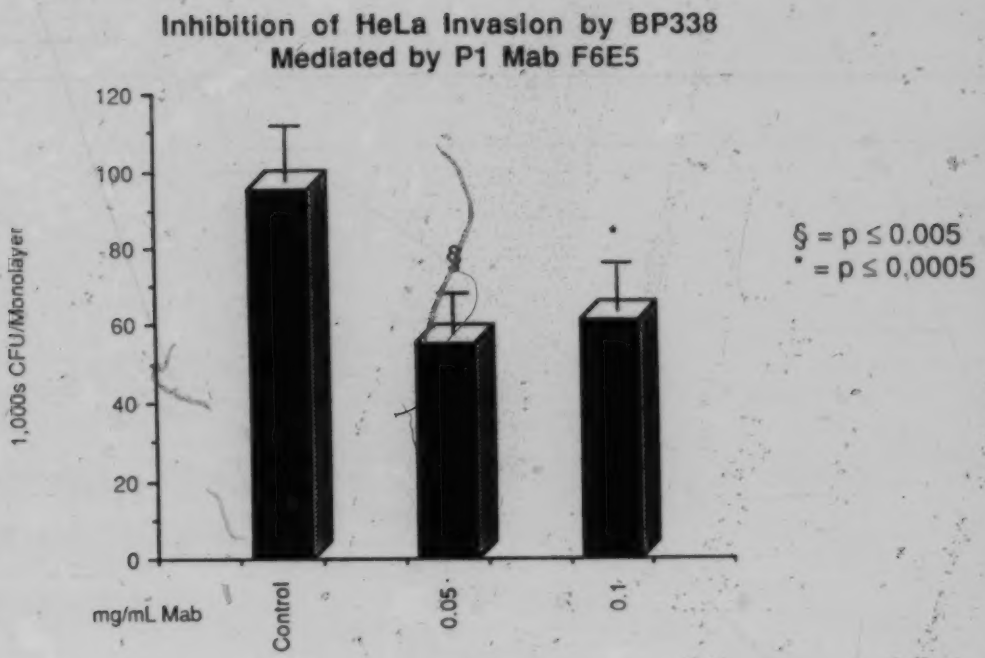
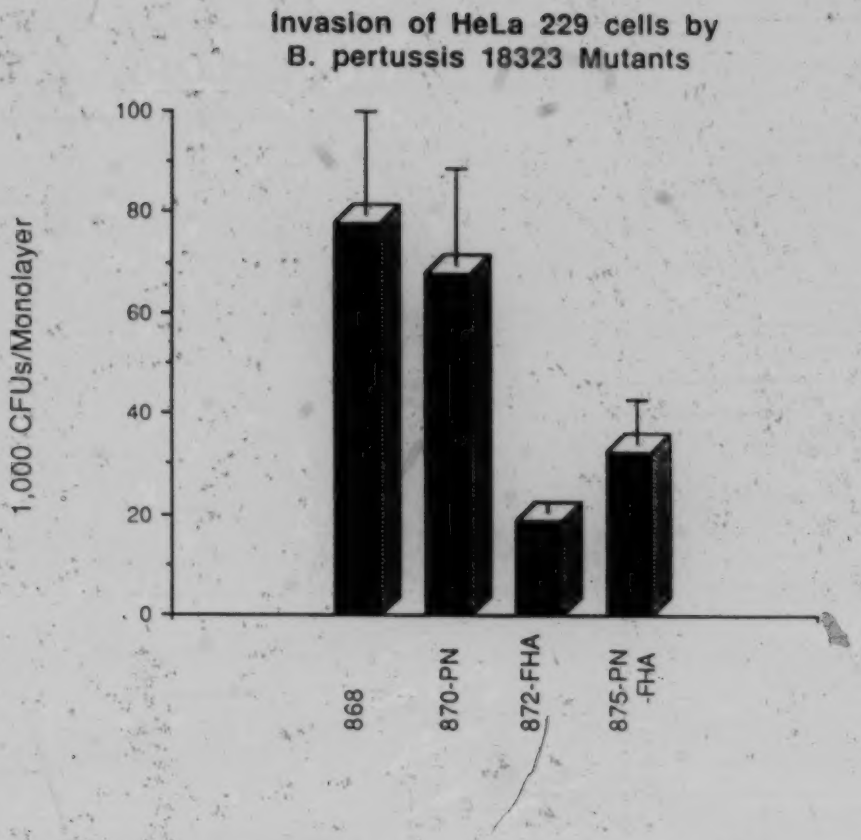

Inhibition of HeLa Invasion by BP338
Mediated by P1 Mab F6E5
1,000s CFU/Monolayer
120
100
80
60
40
20
0
§
*
mg/mL Mab
Control
0.05
0.1
§ = p ≤ 0.005
* = p ≤ 0.0005

FIGURE V.3

Invasion of HeLa 229 cells by *Bordetella pertussis* strain 18323 mutants lacking PN (870), FHA (872), PN and FHA (875) or neither (parent, 868). Each plotted value represents the mean ± standard deviation of polymyxin B-resistant CFUs recovered from three independent experiments.


Invasion of HeLa 229 cells by
B. pertussis 18323 Mutants
1,000 CFUs/Monolayer
100
80
60
40
20
0
868
870-PN
872-FHA
875-PN
-FHA

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Chapter VI.

DEVELOPMENT OF A RAT MODEL FOR RESPIRATORY INFECTION WITH *BORDETELLA PERTUSSIS* AND *BORDETELLA PARAPERTUSSIS*[1]

A. INTRODUCTION

The lack of a suitable animal model for respiratory infection with pertussis is largely responsible for our current lack of understanding regarding the mechanisms of disease pathogenesis and immunity in whooping cough. Although several animal models have been developed, each suffer from drawbacks which limit their applicability.

Among the different laboratory animals tested to date, the mouse respiratory infection model has been the most widely used due to its advantages over other models. The most important advantage it offers is that murine infections mirror natural infections produced in humans in several significant regards, including a higher susceptibility of infant mice to more severe disease, a period of pulmonary infection similar in duration to that of humans, acquired immunity to reinfection, and the occurence of characteristic systemic pathological changes including a pronounced leucocytosis, hypoglycemia, hyperinsulinemia and histamine sensitization (7-9).

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[1] A portion of this chapter has been published. **Woods, D. E., R. Franklin, S. J. Cryz, M. Ganss, M. Peppler and C. Ewanowich.** 1989. Infect. Immun. 57:1018-1024.

However, the model also differs from human disease in several important aspects including a lack of cough and the characteristic whoop, lack of symptomatic respiratory infection, lack of transmission from mouse-to-mouse and the production of lobar pneumonia, which is inconsistent with clinical pathology in human disease.

In the current study, we apply an approach that was originally developed to study chronic respiratory infections caused by *Pseudomonas aeruginosa* (1). This method involves intratracheal instillation of *B. pertussis* encased within agar beads into the left lower lobe of adult male Sprague-Dawley rats. The first report of this model described an unusual pattern of recovery of virulent *B. pertussis* strain Tohama 1 (14). Whereas viable organisms were initally recovered from lung homogenates on days 3 and 7 following initial instillation, on days 10 and 14 following infection viable organisms were not recoverable, but reappeared in lung homogenates at day 21. Examination of lung tissue removed from rats infected with Tohama 1 14 days post-infection by transmission electron microscopy revealed the presence of organisms residing intracellularly. Although it was not possible to discern the particular cell type in which the bacteria were resident, the organisms were structurally intact, electron dense, and most likely viable. Immunofluorescent-antibody treatment of formalin-fixed, paraffin-embedded lung tissue from these animals using commercial antibodies to *B.*

pertussis demonstrated positive fluorescence, which indicated that the intracellular organisms were most likely *B. pertussis*. As a follow-up to this study, we wished to further characterize the intracellular existence of *B. pertussis* within infected rat lungs by quantitating numbers of both total and intracellular *B. pertussis* at various intervals following infection, as well as identifying the cell type(s) which harbored viable intracellular bacteria. In addition, we compared the patterns of recovery of 1) *B. pertussis* mutants lacking expression of single virulence determinants, and 2) a clinical isolate of *B. parapertussis*.

B. MATERIALS AND METHODS

1. Bacterial strains and growth conditions

Bordetella pertussis strains BP338 (parent), BP347 (vir^-), BP353 (FHA^-), and BP357 (PT^-), derivatives of Tohama I previously described in Chapter III, were kindly provided by Dr. Alison A. Weiss, Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, Virginia. *Bordetella parapertussis* strain CN8234N, orginially a clinical isolate, was provided by Mrs. Denise Monack, Deparment of Medical Microbiology, Stanford University, Stanford, California.

Organisms were initally grown on Bordet-Gengou plates containing 13% defibrinated sheep blood at 37°C for 48

hours in a humidified atmosphere. After incubation, suspensions of bacteria were transferred to Stanier-Scholte medium (11) and incubated with shaking (200 rpm in a gyratory shaker; New Brunswick Scientific Co., Inc., Edison, N. J.) for 18-24 hours at 37°C.

2. Preparation of inocula

Beads were prepared by a modification of the method of Cash *et al.* (1). Melted 2% (w/v) Ionagar no. 2 (Oxoid Ltd., London, England) in phosphate buffered saline (PBS), pH 7.0, was kept at 50°C, and a PBS dilution of overnight bacterial culture was added to give a final concentration of approximately 10^7 bacteria per ml of melted agar. Heavy mineral oil (200 ml; Fisher Canada, Inc.) warmed to 50°C was vigorously stirred with a magnetic stir bar, to which was added 10 ml of the bacteria/agar mixture. The oil-agar mixture was cooled rapidly by placing crushed ice around the vessel while stirring continued for approximately 5 minutes. During this time, agar droplets solidified into beads. After three washes in PBS (10,000 x *g*, 4°C) to remove excess mineral oil, the loosely packed beads were suspended in an equal volume of PBS to form a bead-buffer slurry, and the material was placed on ice before inoculation of animals. The final suspension contained beads of markedly varied diameter; those observed in hematoxylin and eosin-stained sections ranged between approximately 50-1,000μM in diameter. Numbers of viable

bacteria present in final bead suspension, which were quantitated on one occasion only, varied between 10^4-10^7 CFU/ml.

3. Infection procedure

Each of thirty-six adult (200-220 grams) male Sprague-Dawley rats were inoculated with one of the five strains listed above. Inoculation was performed by deposition of 0.1 mL of the agar suspension into the lower left lobe of ether-anesthetized and tracheotomized rats using a bead-tipped curved needle as described by Cash *et al.* (1). At intervals of 3, 7, 14 and 31 days following initial infection, groups of rats were sacrificed by ether overdose for quantitative bacteriological and histological examination.

In experiments where lungs were removed exclusively for identification of the cell type(s) harboring *B. pertussis*, rats were inoculated with 10X the standard number of bacteria in order to faciliate the location of cells containing *Bordetella* by TEM.

4. Quantitation of viable total and intracellular bacteria

For quantitative determination of total bacterial counts within infected lungs, the entire lung was removed, and lobes were cut from the hilus and placed in three ml of sterile PBS. The tissue was homogenized with a Polytron

homogenizer (Brinkmann Instruments, Inc,, Westbury, N. Y.) at setting number 6 for 30 seconds. Serial dilutions of the homogenate in PBS were plated on Bordet-Gengou agar plates.

To quantitate viable intracellular bacteria, it was necessary to incubate suspensions of disrupted lung tissue with gentamicin to destroy extracellular bacteria. Lung and heart tissue was first removed *en bloc* above the carina of the trachea. After the heart was excised, the remaining lung tissue was minced with sterile scissors (approximately 200 strokes) into fragments measuring roughly 1 mm x 1 mm. A wash solution containing 136 mM NaCl, 5.3 mM KCl, 5.3 mM glucose, 2.6 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.4), 10 mM HEPES, 1.9 mM $CaCl_2.2H_2O$ and 1.3 mM $MgSO_4$ was added to a final volume of 40 mL. Elastase (Boeringer-Manneheim, Laval, Quebec) was added to a final concentration of 7 U/mL in order to enzymatically disrupt the lung tissue, and DNAse 1, grade II (Sigma Chemical Co., St. Louis, Mo.) was also added to a final concentration of 100µg/mL in order to minimize clumping of disrupted cells by stray DNA. The lungs were then incubated for 30 minutes at 37°C with gentle rocking. After incubation, the rubber tip of a 5 ml syringe barrel was used to express the lung tissue through a stainless steel mesh fused by heat to a plastic support. The resultant cell filtrate was sedimented once (200 x *g*), then resuspended in minimal essential medium (MEM, Gibco Laboratories, Canada) supplemented with 5% fetal bovine

serum (Gibco) and 100µg/ml gentamicin (Gibco). The suspension was incubated for 3 hours at 37°C with gentle rocking in order to allow the gentamicin to destroy extracellar bacteria, then washed three times and resuspended in MEM. Dilutions of the final suspenions were spread onto Bordet-Gengou agar containing 13% sheep blood and 40µg/ml cephalexin (Sigma). The addition of cephalexin was necessary to minimize overgrowth of plates by upper respiratory tract flora which persisted despite gentamicin treatment. However, the presence of 40 µg/mL cephalexin also reduced the growth of *Bordetella* by approximately 50%, as determined by viable count comparisons.

5. Fluorocarbon labelling of phagocytic cells

An emulsion of fluorocarbon/albumin used for perfusion of excised lungs was prepared as follows. Two ml of fluorocarbon FC-75 (3M Co., St. Paul, Minn.) was added to 6 ml of a solution of bovine serum albumin (Sigma, 10 mg/mL in wash solution described above), sonicated for 2 minutes at approximately 75 W (Branson Sonifier, Johns Scientific Co., Calgary, AB), then diluted with an additional 24 ml of the albumin solution. Each pair of lungs was lavaged 3 times with wash solution, then a sufficient volume of fluorocarbon suspension was instilled to inflate lungs to total lung capacity (approximately 8-15 ml/pair). The perfused lungs were immersed in 154 mM NaCl and incubated for 20 minutes at 37°C. After incubation, lungs were

lavaged 8 times with wash solution to remove residual emulsion, then processed for TEM examination.

6. Transmission electron microscopy

Transmission electron microscopic examination of lung sections was performed by Richard K. Sherburne, Department of Medical Microbiology and Infectious Diseases, University of Alberta, Edmonton, Alberta, Canada. Lung tissue was prepared for transmission electron microscopy as previously described (3, Chapters II and III) with modifications. Portions of tissue excised from areas of overt inflammation were fixed with 3.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, at 4°C for 1 hour, washed in cold cacodylate buffer 2 x 15 minutes, and fixed with 1% OsO_4 in cacodylate buffer for 1 hour. The tissue was washed 2 x 15 minutes with buffer, dehydrated with a graded series of alcohol, transferred to propylene oxide for 30 minutes and finally to a 1:1 mixture of propylene oxide and epon for 24 hours unstoppered. This mixture was then replaced with epon and cured at 60°C for 24 hours. Sections of embedded cells were examined using a Philips transmission electron microscope model 410.

7. Histological procedures

Excised lungs were perfused with a solution of 10% (w/v) formalin instilled via the trachea under 10 cm of hydrostatic pressure, while the tissue was immersed in the

same preservative. After fixation, the left lung of each pair was removed below the hilus, cut into two equal pieces across the width of the large lobe, dehydrated in a series of graded alcohols, cleared in xylene and oriented and embedded in paraffin. Embedded lungs were cut sagitally into sections 5 μM thick, dehydrated, cleared, mounted and stained for light microscopy with hematoxylin and eosin.

C. RESULTS

1. Quantitative bacteriological findings

Figures VI.1-5 illustrate total vs. intracellular CFU recovered at 3, 7, 14 or 31 days post-infection from rats infected with either *B. pertussis* or *B. parapertussis*. The virulent parent strain BP338 established a progressive infection in the rat lung (Figure VI.1), in contrast to avirulent mutant BP347 which was essentially cleared within 3 days of initial infection (Figure VI.2). Interestingly, the pattern of recovery of total and intracellular CFU from the lungs of rats infected with BP338 (parent) and BP353 (FHA^-) was strikingly similar (Figure VI. 1 and VI.3). The total number of CFU reached their lowest point at day 14, after which time the values quickly increased in magnitude by a factor of 1,000 to peak again at day 31. Even at 31 days post-infection, significant numbers of viable BP338 and BP353 were present in the lungs of infected rats. In contrast to the pattern of recovery of total CFU, numbers

same preservative. After fixation, the left lung of each pair was removed below the hilus, cut into two equal pieces across the width of the large lobe, dehydrated in a series of graded alcohols, cleared in xylene and oriented and embedded in paraffin. Embedded lungs were cut sagitally into sections 5 μM thick, dehydrated, cleared, mounted and stained for light microscopy with hematoxylin and eosin.

C. RESULTS

1. Quantitative bacteriological findings

Figures VI.1-5 illustrate total vs. intracellular CFU recovered at 3, 7, 14 or 31 days post-infection from rats infected with either *B. pertussis* or *B.parapertussis*. The virulent parent strain BP338 established a progressive infection in the rat lung (Figure VI.1), in contrast to avirulent mutant BP347 which was essentially cleared within 3 days of initial infection (Figure VI.2). Interestingly, the pattern of recovery of total and intracellular CFU from the lungs of rats infected with BP338 (parent) and BP353 (FHA^-) was strikingly similar (Figure VI. 1 and VI.3). The total number of CFU reached their lowest point at day 14, after which time the values quickly increased in magnitude by a factor of 1,000 to peak again at day 31. Even at 31 days post-infection, significant numbers of viable BP338 and BP353 were present in the lungs of infected rats. In contrast to the pattern of recovery of total CFU, numbers

of intracellular BP338 and BP353 reached a peak at day 7, and decreased thereafter up to day 31.

The pattern of recovery of BP357 (PT-) was similar to that of BP338 and BP353, where total numbers reached a minimum at day 14 and sharply increased thereafter, although on an approximately 10-fold lower scale (Figure VI.4). Unlike BP338 and BP353, however, numbers of intracellular bacteria peaked slightly earlier, at day 3, then steadily decreased up to day 31.

The recovery of total *B. parapertussis* CFUs from infected lungs differed from recovery of *B. pertussis* in both pattern and magnitiude (Figure VI.5). Numbers of both total and intracellular CFU recovered from the lungs of rats infected with *B. parapertussis* were exceptionally high compared to numbers of *B. pertussis* recovered. In addition, numbers of total CFU of *B. parapertussis* declined steadily beginning at day 3 and did not exhibit the same sudden decrease and increase seen with the *B. pertussis* strains.

2. Histopathological findings

The histopathological features of infection with virulent Bordetellae were described in an earlier report by Woods *et al.* (14). No significant differences in degree or progression of pathological changes were observed between the strains tested, with the exception of BP347 which was cleared within 3 days PI and thus did not elicit lung pathology. At day 3, a mild lymphocytic infiltrate

appeared in the bronchi, accompanied by peribronchial lymphoid hyperplasia. By day 7, a necrotizing inflammation of the tracheobronchial mucous membranes was noted, characterized by both mononuclear and polymorphonuclear cells, which spread to the terminal bronchioles and alveoli by day 21. In the sections examined for this study, subpleural atelectasis, thickening of alveolar septae, and consolidated areas of pronounced inflammation and polymorphonuclear leukocytic infiltration were commonly observed in sections representing basal areas of the lung. Pathological changes were only rarely observed in apical lung areas (ie. upper half of lung). No significant differences in degree or progression of pathological changes were observed between the strains tested.

3. TEM examination of lung tissue infected with BP338

Lung tissues were recovered from rats infected with ten times the usual inoculum of BP338 in order to reduce the time required to locate intracellular organisms by TEM. Excised lungs were initially perfused with a suspension of fluorocarbon in order to label phagocytic cell types *in situ*, then processed as usual for TEM examination. In the event that a phagocytic cell type harbored intracellular *B. pertussis*, it could be identified by the presence of fluorocarbon inclusions engulfed during incubation with the fluorocarbon. Figure VI.6 illustrates the characteristic

TEM appearance of fluorocarbon within an unidentified phagocytic cell type, which appear as electron-lucent spheres (large arrowheads). The cell shown in Figure VI.6 also contains several intracellular bacteria which appear to be structurally intact, electron-dense, and therefore most likely viable (small arrowheads).

D. DISCUSSION

In this model for respiratory infection with *Bordetella*, bacteria encased within agar beads were instilled directly into the base of the rat lung via tracheotomy. Direct instillation alleviates the requirement for adhesins which normally mediate adherence of *Bordetella* to mucosal surfaces of the upper respiratory tract, which represent the native site for initial colonization by these organisms in humans. Consequently, it was not surprising to find no apparent difference between recovery of parent BP338 and mutant BP353 lacking FHA, an important adhesin for adherence of *B. petussis* to ciliated cell tufts (12, 13).

Whereas a lack of FHA expression did not significantly affect recovery of *B. pertussis*, the lack of PT in strain BP357 resulted in the recovery of significantly fewer organisms compared to the parental control (>10-fold reduction in recovery). This observation may be explained by previous reports of inhibition of macrophage migration

(4-6) and neutrophil, lymphocyte and macrophage chemotaxis by PT (10). If PT arms *B. pertussis* with the ability to inhibit phagocytic influx, then strain BP357 lacking in expression of PT would be more efficiently cleared from the lungs of infected rats than strains BP338 and BP353. This would be reflected by a lower CFU recovery early on in the infection.

For reasons unknown, numbers of both total and intracellular *B. parapertussis* recovered from infected rat lungs were surprisingly high compared to recovery of *B. pertussis*. It should be determined whether this difference is consistent by first repeating the experiment to rule out differences in inoculum viability. If the trend is consistent, then it would be interesting to subject several different clinical isolates of *B. parapertussis* to the assay to determine whether the effect is strain-dependent.

This experiment was terminated at 31 days post-infection, when the lungs of rats infected with all strains tested except BP347 still contained viable bacteria (both extra- and intra-cellular). As the behavior of *Bordetella* within the lung during extended periods (ie. past 31 days post-infection) is especially relevant to the question of intracellular carriage and persistance, future experiments should be continued until viable *Bordetella* are no longer recovered.

In view of previous data which indicated the survival of *Bordetella pertussis* within alveolar macrophages (2) and

polymorphonuclear leukocytes (R. L. Friedman, L. L. Steed and M. Setareh, *Abst Ann Meet Amer Soc Microb*, 1988, p. 200), it was possible that viable *Bordetella* were maintained within alveolar phagocytes. In order to investigate this possibility, we used an emulsion of fluorocarbon and albumin which would be engulfed by phagocytic cells upon introduction into excised lungs by perfusion. By virtue of its characteristic appearance, fluorocarbon is easily identified by transmission electron microscopic examination, and was therefore used to label phagocytic cells *in situ* prior to processing of lung tissue for TEM examination. Several cells like the one depicted in Figure VI.6 containing both intracellular bacteria and fluorocarbon were observed. The presence of the fluorocarbon identifies the cell as either a macrophage or neutrophil. We were unable to confirm the identity of the intracellar bacteria as *B. pertussis* using immunogold techniques because the cells were initially fixed in a solution of 3.5% glutaraldehyde, which destroys the antigenic character of most epitopes. However, because alveolar areas of the lung are normally kept sterile by non-specific defenses of the upper respiratory tract, we feel that it is likely that these bacteria are *B. pertussis*.

As noted in the results, no remarkable differences between pathological findings were observed among lungs infected with strains BP338, BP357, BP353 or *B.*

parapertussis. It should be mentioned, however, that lungs were sectioned randomly without prior selection of grossly inflammed areas. Differences may have been apparent if the interior of the lungs had been examined visually for areas of inflammation and oriented correctly for embedding and sectioning.

In summary, this chapter describes a novel model for respiratory infection with *Bordetella* which provides support for the intracellular survival and persistance of both *B. pertussis* and *B. parapertussis in vivo*. The natural site for intracellular persistance of *Bordetella* is not yet known. It is equally possible that it is in a lower respiratory compartment or in the mucosa of the upper respiratory tract, or a combination of both. This model concentrates specifically on the behavior of *Bordetella* within the alveolar compartment of the lung. It does not examine the possibilty of intracellular persistance within the upper respiratory tract, which still remains to be evaluated. In addition, the effect of the agar encasement on dissemination of the bacteria in the midst of a pronounced neutophil infiltrate needs to be examined. As previously mentioned, the beads used in these experiments were of widely varying diameters, and, on average, extremely large (up to 1 mm, visible macroscopically in stained sections). The sheer size and physical presence of these beads in the lower alveolar regions of the lungs likely creates undesirable artifacts such a disruption of

normal pulmonary function and atelectasis secondary to airway obstruction. These experiments should be repeated using beads of a much smaller diameter (eg. 10-20 μM) in order to more accurately assess the usefulness of the model for studying respiratory infection with *Bordetella*.

FIGURE VI.1

Viable counts of BP338 (virulent parent) in rat lung. Plotted points represent mean ± standard error for 3 rats.

Viable Counts of BP338 (Vir+ parent) in Rat Lung

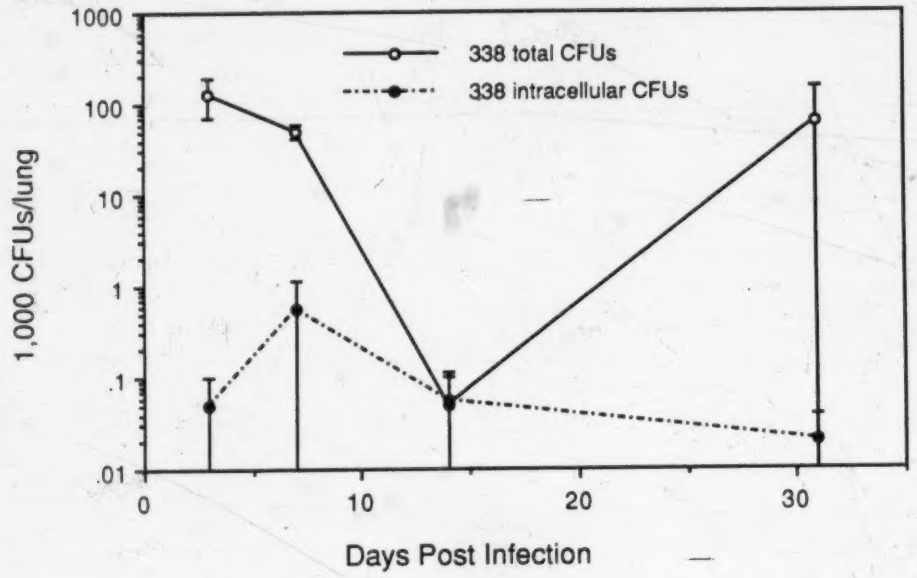


FIGURE VI.2

Viable counts of BP347 (Vir^-) in rat lung. Plotted points represent mean ± standard error for 3 rats.

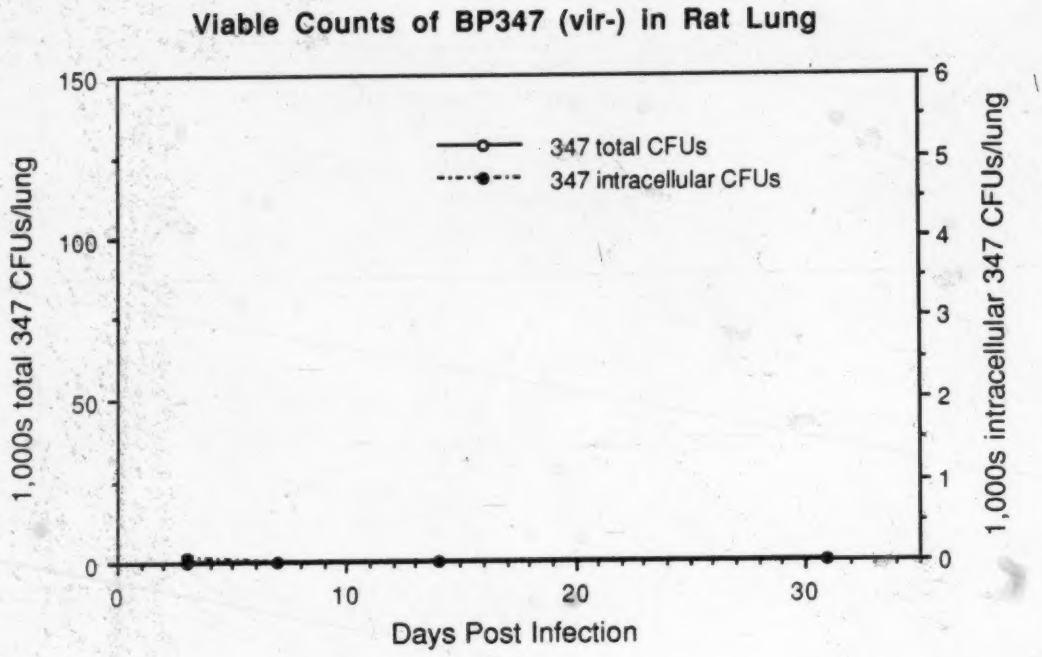

Viable Counts of BP347 (vir-) in Rat Lung
1,000s total 347 CFUs/lung
1,000s intracellular 347 CFUs/lung
347 total CFUs
347 intracellular CFUs
150
100
50
0
6
5
4
3
2
1
0
0
10
20
30
Days Post Infection

FIGURE VI.3

Viable counts of BP353 (FHA^-) in rat lung. Plotted points represent mean ± standard error for 3 rats.

Viable Counts of BP353 (FHA-) in Rat Lung

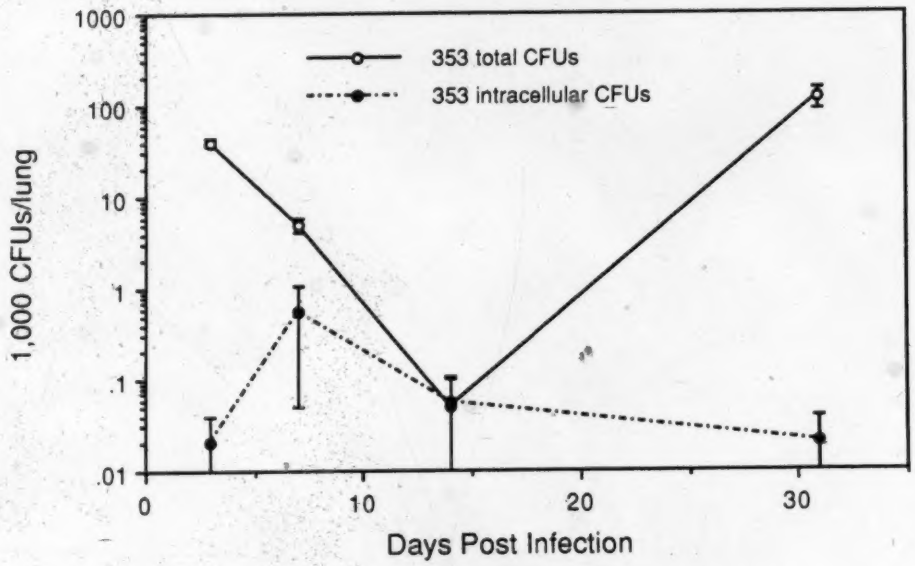


FIGURE VI.4

Viable counts of BP357 (PT^-) in rat lung. Plotted points represent mean ± standard error for 3 rats.

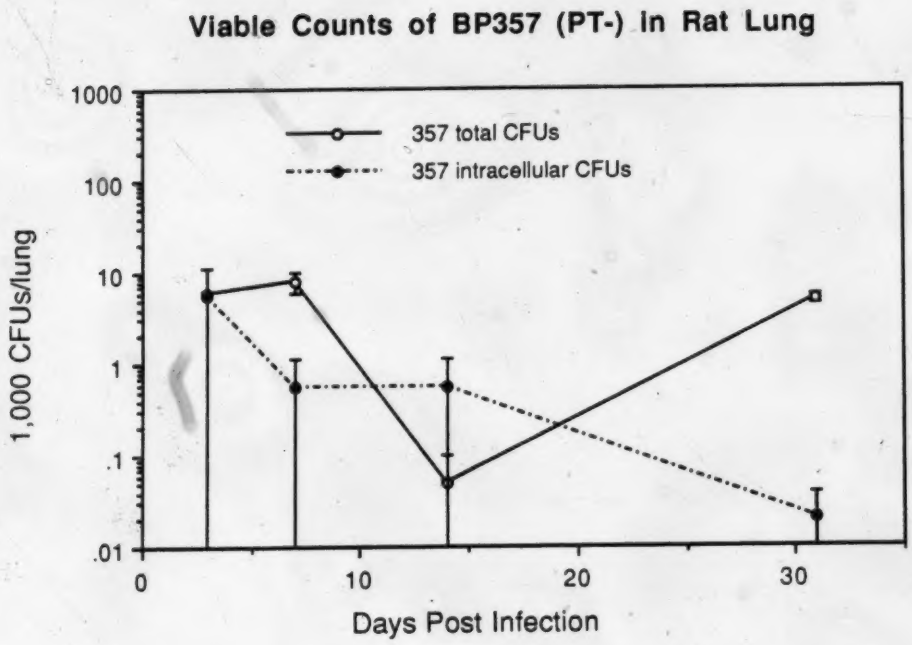

Viable Counts of BP357 (PT-) in Rat Lung
357 total CFUs
357 intracellular CFUs
1000
100
10
1
.1
.01
1,000 CFUs/lung
0
10
20
30
Days Post Infection

FIGURE VI.5

Viable counts of *B. parapertussis* in rat lung. Plotted points represent mean ± standard error for 3 rats.

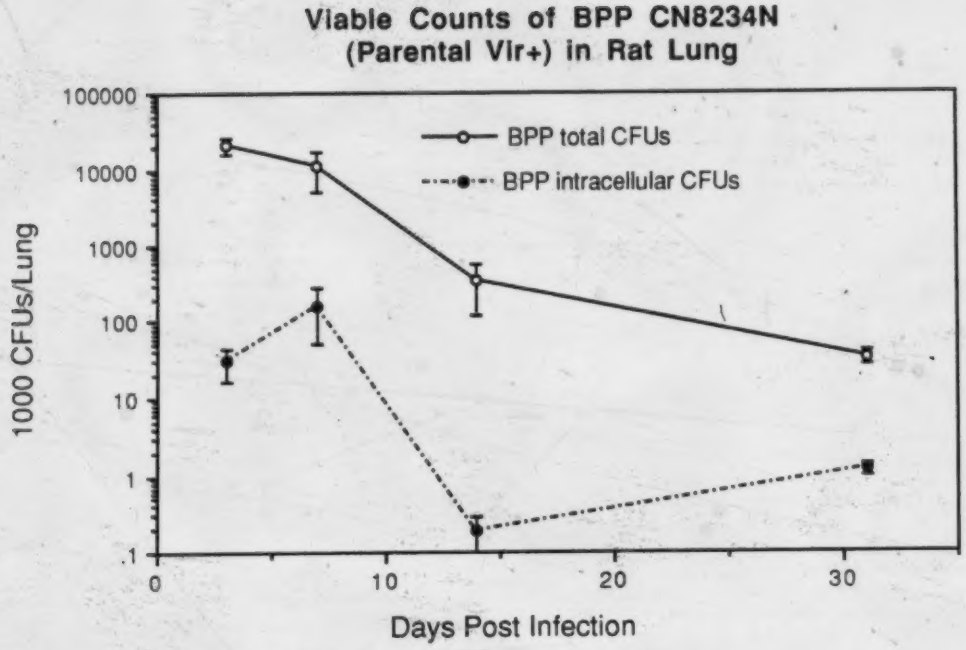
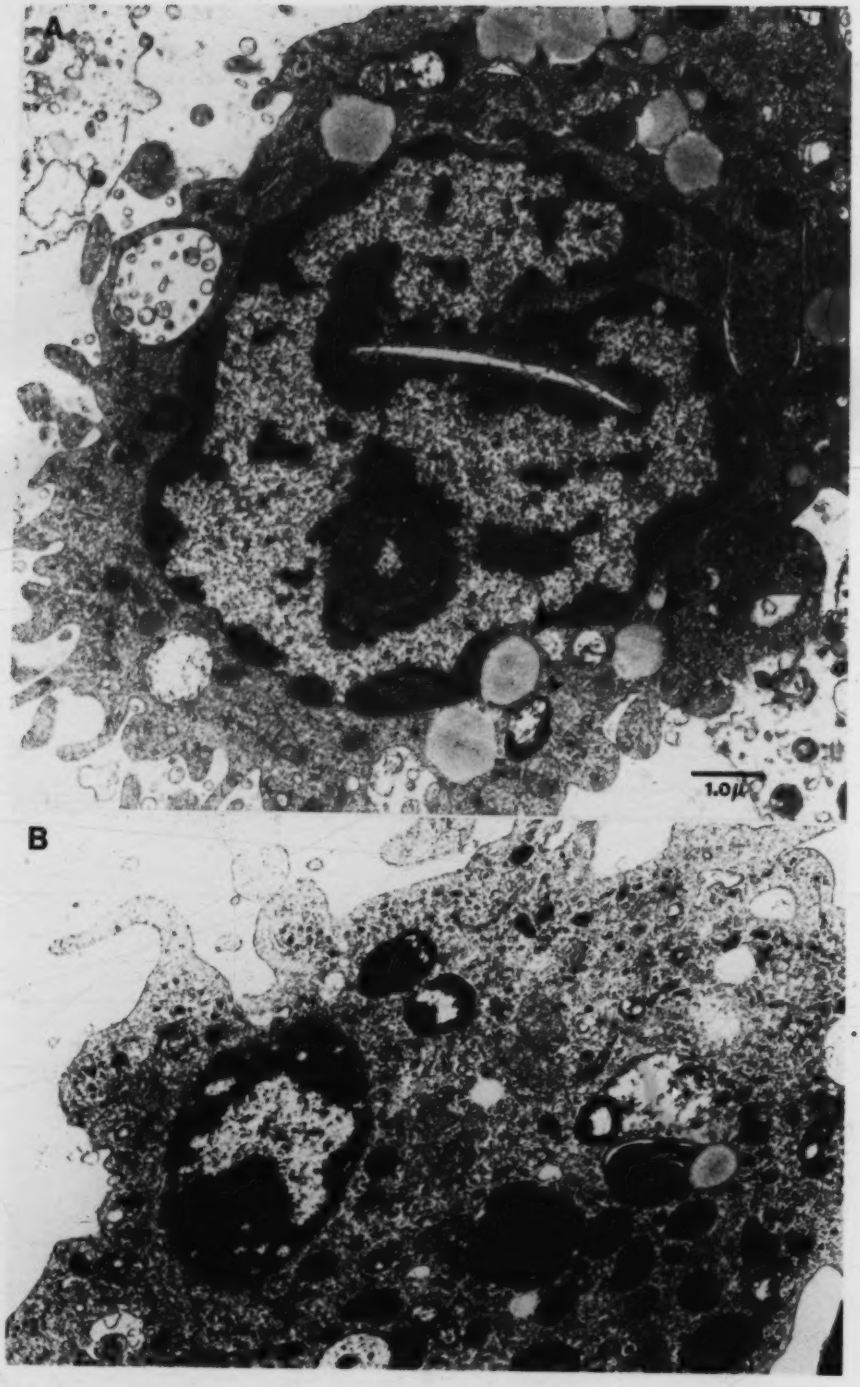

Viable Counts of BPP CN8234N
(Parental Vir+) in Rat Lung
100000
10000
1000
100
10
1
.1
1000 CFUs/Lung
BPP total CFUs
BPP intracellular CFUs
0
10
20
30
Days Post Infection

FIGURE VI.6

Transmission electron microscopic view of alveolar lung tissue recovered 3 days after intratracheal inoculation with BP338. Phagocytic cells were labelled *in situ* with a fluorocarbon/albumin suspension. Cell depicted contains multiple fluorocarbon spheres (large arrowheads) indicating its phagocytic capacity, in addition to several structurally intact, electron-dense bacteria which are presumably *B. pertussis* (small arrowheads).


A
1.0μ
B

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CHAPTER VII

DISCUSSION

The findings described in this thesis provide a preliminary framework for further study of the subject of invasion of mammalian cells by species of *Bordetella*. Many questions still exist, and there is still much to be learned. When our investigation began, only very limited information was available on the subject of *Bordetella* invasion. Therefore, it was necessary to first critically evaluate the organisms for evidence of invasive ability. Once we were completely convinced that the phenomenon was real and not an artifact produced by experimental methods, our efforts were directed toward obtaining solid data which would also convince others of its validity. Consequently, our research has centered upon the initial steps of invasion, ie. adherence and internalization. We have not examined long-term survival and mechanisms for evading cellular killing once internalized. These are important questions related to *Bordetella* carriage in the population.

The following chapter provides a better perspective of *Bordetella* invasiveness by reviewing it within the context of invasive strategies of other facultative intracellular bacterial pathogens. Where appropriate, logical avenues for further research are indicated.

A. ADHERENCE AND ENTRY

1. Adherence

Like most nonphotosynthetic life forms, *Bordetella sp.* must parasitize living creatures in order to obtain food for survival. Because they are unable to survive outside the mammalian host, *Bordetella* express various attachment factors which allow them to succesfully colonize the host. In so doing, they escape prompt removal by non-specific clearance mechanisms. Whether expression of virulence determinants which mediate adherence is constitutive or induced by unknown factors in the changed environment of the host is currently a topic of intense research effort, as outlined in Chapter I. However regulated, adhesins allow the organisms to successfully colonize their host so they may begin to compete for nutritional factors required for survival.

In the case of *B. pertussis*, adherence to mammalian cells is multifactorial. At least four different attachment factors have been implicated, depending on the system in question, including FHA (14, 27, 38), PT (14, 37) fimbrial agglutinogens (14, 27, 33), and pertactin (22).

2. Role of the host cell in bacterial invasion

Once established on a mucosal surface, a pathogen can either remain localized on the exterior surfaces or proceed to invade the host cell. Although an intracellular

lifestyle offers several advantages, including protection from specific- and non-specific host immune defenses and freedom from competition with normal flora for available nutrients, it is not without its disadvantages. For example, the pathogen must first have made some sort of evolutionary adaptation which permits it to evoke its own uptake by the host cell, most of which are epithelial in origin and therefore lack specialized phagocytic pathways. Once the pathogen has invaded a nonprofessional phagocyte, it must also possess strategies to avoid intracellular destruction by the host cell.

The process of entry into host cells by most invasive bacterial pathogens appears to occur primarily by pre-exisiting pathways, and requires active participation on the part of both the bacterial and host cell. The component of the host cell usually involved in uptake is the actin cytoskeleton, as evidenced by the observation that invasion of many pathogens is inhibited with microfilment inhibitors cytochalasins B and D; including *Shigella* (15), *Salmonella* (8), *Yersinia* (3) and *Bordetella* (Chapter II and III). In some cases, entry is accompanied by a rearrangement of cytoskeletal actin filaments in areas proximal to the attachment site (6, 11), as demonstrated by microscopic examination of infected cells with fluorescent derivatives of phalloidin, a bicyclic heptapeptide which binds to actin filaments (7).

One organism which does not appear to utilize an endocytic pathway involving actin microfilaments is *Chlamydia psittaci*. In an ultrastructual study, Hodinka and Wyrick (16) describe the association between invading *C. psittaci* and clathrin-coated pits, which are specialized plasma membrane domains which internalize molecules required by the cell in a ubiquitous mechanism known as receptor-mediated endocytosis (RME) (13). Clathrin was not observed in association with membrane areas surrounding *Bordetella* in the process of uptake. Furthermore, monodansylcadaverine, a reagent which has been reported to inhibit RME by inhibition of enzymes which stabilize receptor-ligand clustering in coated pits (34), did not inhibit invasion of HeLa cells by *Bordetella parapertussis*, as shown in chapter II. We therefore excluded RME as a significant means of entry of *Bordetella*.

3. Bacterial determinants of host cell invasion

Invasive bacterial pathogens appear to express several types of surface-exposed factors which mediate self-induced uptake after they interact with appropriate receptors on the host cell. Although the genes encoding several of these factors have been identified in both plasmid and chromosomal locations, less is known of their corresponding protein products. One such protein which has been relatively well-characterized is invasin, a 103 kDa product of the *inv* gene (17). Invasin mediates adherence of

Yersinia pseudotuberculosis to integrin receptors of mammalian cells by an RGD-independent mechanism (18). The 14 kDa protein product encoded by a second invasion locus of *Yersinia*, designated *ail* (adherence *i*nvasion *l*ocus), promotes invasion of various types of mammalian cells by *Yersinia enterocolitica* (24). The invasion determinant of *Shigella flexneri* is actually a complex encoded by a cluster of "invasion plasmid antigen" or *ipa* genes located on a 140 MDa plasmid (1, 2, 4, 23, 32), whose expression is regulated by the product of a gene downstream from the cluster designated *virF* (29). Invasion determinants of *Salmonella* are chromosomally-encoded, and appear to be expressed *de novo* following interaction with epithelial cell surfaces (12). Six classes of Tn*pho*A mutants deficient in the ability to enter eucaryotic cells have recently been identified (10). Elucidation of their defects will help to identify and characterize *Salmonella* invasion determinants.

Invasion determinants of *Bordetella* are more obscure. Data presented in chapter III indicates that both FHA and PT are required for invasion of *B. pertussis*. However, since they are commonly regarded as adhesins (27, 33, 37), we presume that they are most likely involved in adherence instead of the actual process of invasion, since a reduction in adherence would logically reduce invasion. Using a different procedure for quantitating invasion of HeLa cells *B. pertussis*, Lee *et al.* reported that a mutant lacking FHA was not inhibited in its ability to invade

(21). This observation is difficult to explain in light of published reports describing a role of FHA as an adhesin for HeLa cells (27, 33), although methodological differences may be at least partly responsible. For example, polymyxin B is used in place of gentamicin in the standard assay to select against extracellular organisms. This amphipathic molecule possesses a net positive charge due to the presence of a dibutanoic moiety, in addition to a nonpolar acyl tail which intercalates between fatty acid components of biological membranes as depicted in Figure VII.1. By virtue of the physical properties which mediate the interaction between polymyxin B and HeLa membranes, the presence of polymyxin B may be sufficient to change the character of the HeLa membrane in such a way that artifacts may be introduced into the assay. This assay also uses glass beads to disperse HeLa monolayers, which are subsequently sonicated to break open the cells and release intracellular bacteria. These unusual treatments may also be a potential source of artifacts producing descrepancies between data obtained from the two assay types.

Within the panel of Tn*5* *lac* mutants which we examined in Chapter III, two were significantly reduced in their ability to invade HeLa cells. The products of the loci disrupted by the presence of the transposon in these mutants are unknown, although they are currently under examination in the laboratory of Dr. A. Weiss.

Based on data presented in Chapter V, one surface protein which appears to be involved in internalization is pertactin. Because adherence and invasion are inextricably linked, it is difficult to assign a role for pertactin in either process exclusively. However, in view of data presented in Chapter V which indicate the importance of the first RGD sequence in pertactin, it is tempting to speculate that an interaction between pertactin and an integrin receptor on the HeLa cell surface promotes penetration of *B. pertussis*. Further characterization of the corresponding receptor on the surface of the mammalian cell recognized by pertactin is clearly indicated.

A role for integrins in the phagocytosis of *B. pertussis* has recently been demonstrated by Relman *et al.* (28), who described a specific interaction between integrin complement receptor CR3 on human macrophages and an RGD sequence within FHA. As discussed in Chapter V, CR3 is a prudent choice for a receptor on the part of the bacteria because ligation of CR3 induces internalization but does not activate the mechanisms which initiate intracellular killing. Indeed, previous data presented by Friedman *et al.* (R. L. Friedman and P. Z. Detskey, Abst. Annu. Meet. Am. Soc. Microbiol. 1989, D128, p. 103) in addition to our own unpublished observations indicates that *B. pertussis* is capable of survival and multiplication within human monocytes and monocyte-derived macrophages.

B. SURVIVAL WITHIN THE HOST CELL

Once internalized, a facultative intracellular pathogen must provide its own means to escape killing within the potentially threatening environment of the host cell. Professional phagocytes exhibit a number of microbicidal activities including generation of cell wall-active hydrogen peroxide and superoxide radicals, activation of myeloperoxidase, lysosomal fusion and the subsequent release of lytic enzymes, and destruction by lysozyme, lactoferrin, and defensins. Of these, non-professional phagocytes likely possess only lysosomal enzymes for defense against invading organisms, which is why successful intracellular bacteria pathogens have evolved several strategies to avoid the lethal consequences of activation of lysosmal enzymes following phagosome-lysosme fusion. As outlined by Moulder in a thorough review of intracellular parasitism (25), these include adaptation to a host cell without lysosomes, escape from the phagosome, resistance to lysosomal enzymes and prevention of lysosomal fusion.

Shigella spp. possess a contact hemolysin which is thought to lyse the phagosomal membrane surrouding the bacterium (30), as does *Listeria monocytogenes* (20). Rapid intracellular multiplication and lysis of the host cell ensue, allowing Shigellae to infect neighboring host cells (31). While *Shigella spp.* remain localized at epithelial

surfaces and induce inflammation only as deep as the underlying lamina propria, *Salmonellae* must cross the epithelial barrier in order to reach deeper target tissues and disseminate within the host. This is accomplished in a process termed "transcytosis" where a bacterium enclosed in a phagosome created at the apical membrane of the host cell traverses the cytosol and is released at the basal face following fusion between phagosomal and host cell membranes. (9, 35). Both *Salmonella* and *Yersinia spp.* utilize cells of the reticuloendothelial system to expediate their dissemination within the host, and have consequently evolved sophisticated means to evade killing by the elaborate defense strategies of professional phagocytes. For example, *Yersinia pestis* appears to impair the generation of oxygen radicals by inhbition of the oxidative burst which normally follows phagocytosis (5).

The intracellular survival strategies of *Bordetella* have not yet been documented. TEM examination of *Bordetella* within both nonprofessional and professional phagocytes indicates that the organisms remain within tight endocytic vesicles, and do not appear to escape into the cytoplasm (Chapter II and III, R. L. Friedman and P. Z. Detskey, Abst. Annu. Meet. Am. Soc. Microbiol. 1989, D128, p. 103). It is possible that *Bordetella* have evolved a means to prevent phagosome-lysosome fusion in order to thwart destruction by lysosmal enzymes. If so, they must also possess a means to obtain nutrient intermediaries

across a host-cell derived phagosomal membrane in order to survive. Perhaps the same modification of the phagosomal membrane effected by the organisms contained within prevents lysosomal fusion and also facilitates acquisition of host cell-derived nutrients.

An important question which remains to be answered is whether the intracellular environment possessess the appropriate signals to induce antigenic modulation of *Bordetella* to an avirulent phenotype. As outlined in Chapter 1, antigenic modulation to an avirulent phenotype represses the synthesis of the *vir* gene product, a positive regulatory element. In the absence of the *vir* gene product, the expression of a set of genes whose expression is *vir*-activated is repressed, while synthesis of a set of proteins whose expression is *vir*-repressed is derepressed, ie. activated (19). While the functions of the *vir*-repressed protein products are unknown as yet, it is tempting to speculate that they may confer survival properties to intracellular *Bordetella*.

We used a set of *vir*-repressed Tn*phoA* mutants to address the question of whether antigenic modulation followed transition to an intracellular environment by assaying HeLa monolayers for expression of alkaline phosphatase once the mutants had established themselves intracellularly. However, we were unable to completely extinguish endogenous HeLa cell alkaline phosphatase activity using a variety of specific and non-specific

inhibitors. In addition, each of the six *vir*-repressed mutants we tested expressed alkaline phosphatase in limited amounts even when they expressed a virulent phenotype. Such technical difficulties prevented us from uncovering evidence of intracellular modulation.

One possible strategy which could be used to approach this question is to create mutations in *vir*-repressed genes using the transposon Tn5 *luxAB* transferred into the chromosome of *B. pertussis* following conjugation with a suicide plasmid. Tn*5 luxAB* encodes a luciferase enzyme which catalyzes the formation of photons from a reaction involving an aldehyde and a flavin mononucleotide (26). The virtue of using this reporter gene is the exquisite sensitivity with which its product can be detected - using the appropriate instrumentation, it is theoretically possible to detect a single bacterial cell expressing the luciferase enzyme (26) The occurrence of antigenic modulation within an intracellular environment could be examined by monitoring HeLa monolayers infected with a strain expressing the luciferase enzyme under *vir*-repressed conditions for photon emission at specific intervals following inoculation. However, if the expression of *vir*-repressed genes normally occurs in limited amounts within *Bordetella* expressing a virulent phenotype (ie. a "leaky" phenotype), as described above for the Tn*phoA* mutants, the use of Tn*5 luxAB* as a reporter gene would be similarly limited.

The ability of *B. pertussis* to modulate within an intracellular environment would confer several advantages to an intracellular pathogen. First, synthesis of *vir*-activated toxins would be repressed, thus minimizing disruption of the host cell. Additionally, as the transcription of *vir*-activated genes are repressed following modulation, the synthesis of *vir*-repressed genes is deprepressed. The protein products of these *vir*-repressed genes may facilitate intracellular survival and persistence of *Bordetella*. In short, antigenic modulation would allow the organisms to remain relatively quiescent within the protected environment of their host cells, effectively invisible to the continuous immune surveillance taking place within the extracellular milieu.

Unlike several intracellular pathogens which undergo unrestricted intracellular multiplication inducing cell lysis, *Bordetella* do not replicate extensively within the host cell and must therefore rely on more subtle means of release. Possible mechanisms include synthesis and accumulation of a proteolytic enzyme that weakens the plasma membrane (a product of a *vir*-repressed gene?); a return to the virulent state induced by changes in the intracellular environment which accompany aging of the host cell, leading to cell death and bacterial release as a result of *vir*-activated toxin action, release by exocytosis (stimulus?), or the accumulation of osmotically active substances within the phagosome enclosing the bacterium,

leading to cell lysis. Following release from the host cell, *Bordetella* would undergo cell-to-cell or host-to-host transit, thereby permitting bacterial persistance or perpetuating the infection, respectively.

C. ANIMAL MODELS FOR *BORDETELLA* INVASION

Despite the convenience offered by cell culture models for studies of bacterial invasion, they posses several inherent limitations (25). The pertinence of information obtained using cell culture models is limited by such factors as inflated multiplicities of infection created by artificially large inocula of bacteria, unrepresentative cell density, and a lack of variation in representative cell types and modifying influences of hormones, growth factors and antibodies. The use of explanted tissue such as tracheal rings offers several benefits over continuous cell cultures including the presence of representative cell types in the correct ratios and a native tissue architecture. However, the interactions between bacteria and tissue within a representative setting in the presence of appropriate modifying influences cannot be properly assessed unless tested *in vivo*. With such limitations in mind, it was therefore necessary for us to determine whether the invasive behavior of *Bordetella* observed in HeLa 229 monolayers and nasal turbinate primary epithelial cell cultures similarly occurs *in vivo*.

We examined *Bordetella* for evidence of invasion and intracellular survival in the rat model described in Chapter VI. Viable intracellular organisms from all strains tested were recovered from lung tissue as late as 31 days post-infection. TEM examination of lung sections recovered at 3 days post-infection revealed the presence of apparently viable *B. pertussis* within an unknown phagocytic cell type. The phagocytic nature of this cell type was identified by the presence of intracellular fluorocarbon spheres which were used to label phagocytes *in situ* following removal of the lungs from the rat.

We also attempted to assess invasive behavior of *B. pertussis* in a murine model of infection based on the results of a thesis published in 1981 by Lewis Tomalty (M. Sc. thesis, Univeristy of Alberta, Edmonton, Alberta, 1981). Tomalty examined the humoral responses of adult female ICR mice to intranasal *B. pertussis* infection over a six month interval. He found that while viable counts of *B. pertussis* were negative by day 44 post-infection, *B. pertussis* persisted in the lung up to day 129 following infection as detected by immunofluoresence. Futhermore, subclinically-infected mice harboring fluorescence-positive bacteria were capable of transmitting *B. pertussis* to uninfected cage mates as evidenced by seroconversion and the appearance of fluorescence-positive organisms within lung impression smears.

Based on these provocative findings, we attempted to repeat Tomalty's findings in order to determine whether the uncultivatable organisms were harbored in an intracellular compartment, as Tomalty had speculated in his discussion. However, using virulent phase I Tohama organisms we were unable to achieve a productive infection in adult mice, despite various inocula (up to 10^{10}/mouse) and volumes of inocula (10-25µl intranasally) tested. Perhaps the use of a more virulent *B. pertussis* strain for mice would permit colonization so that recovery over a prolonged period could be studied. In view of Tomalty's data, such experiments certainly bear repeating.

D. CONCLUSIONS

While many researchers endeavor to study disease caused by *B. pertussis*, perhaps it is equally important to study infection. We may have a reasonable understanding of which virulence determinants are important for pathogenesis of *Bordetella* from the standpoint of disease production, but our knowledge of determinants important for invasion and persistance is extremely limited. This is an important consideration for vaccine formulation. Without a clear understanding of the interrelationships between virulence factors of *Bordetella* which mediate infection *and* disease, perhaps we are too hasty in our attempt to select those which should be included in subunit vaccines, and

those which are unimportant. Formulation of a completely efficacious subunit vaccine must await a complete and thorough understanding of disease pathogenesis, including the potential role of invasion and intracellular survival in carriage of *Bordetella*.

FIGURE VII.1

Interaction between the amphipathic polymyxin B molecule and a biological membrane.

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From: Storm, D. R., K. S. Rosenthal, and P. E. Swanson. (1977) Polymyxins and related peptide antibiotics. *Ann Rev Biochem* 46:723-763.

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